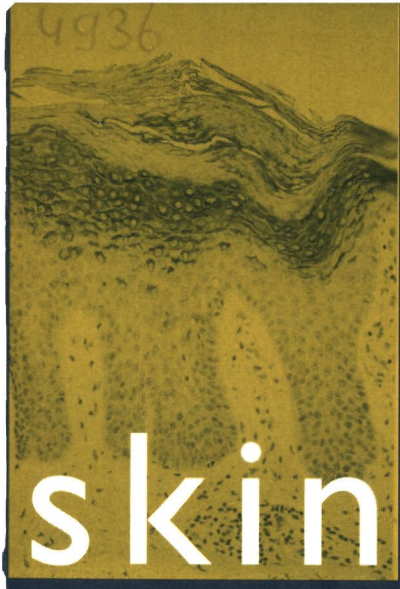

skin-derived antileukoproteinase

EXPRESSION PATTERNS AND CLINICAL APPLICATIONS

HANS ALKEMADE

SKIN-DERIVED ANTILEUKOPROTEINASE

expression patterns and clinical applications

SKIN-DERIVED ANTILEUKOPROTEINASE

expressie patronen en klinische toepassingen

Drukkerij: De Kleijn, Wijchen.

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Een wetenschappelijke proeve op het gebied
van de Medische Wetenschappen

Proefschrift
ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
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Johannes Adrianus Cornelis Alkemade
geboren op 18 juni 1959 te Noordwijk

Promotor : Prof. dr. P.C.M. van de Kerkhof
Co-promotor : Dr. J. Schalkwijk

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Voor mijn ouders,
voor ome Jan,
en ter herinnering aan tante Gré

Manuscriptcommissie: Prof. dr. D.J. Ruiter (voorzitter)
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CONTENTS

ABBREVIATIONS

ALP:	antileukoprotease
Da:	dalton
Ig:	immunoglobulin
PCR:	polymerase chain reaction
PVDF:	polyvinylidene difluoride
PMN:	polymorphonuclear leukocytes
RT:	reverse transcriptase
SDS-PAGE:	sodiumdodecylsulfate polyacrylamide gelelectrophoresis
SKALP:	skin-derived antileukoproteinase
SLPI:	secretory leukocyte proteinase inhibitor
Tricine:	*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine

Chapter 1

Preface and introduction

PREFACE

This thesis is the result of a four-year project funded by the Faculty of Medical Sciences, University of Nijmegen. In this project the author was appointed as 'assistent-in-opleiding (AIO)' (junior researcher) from 01.01.91 to 01.01.95. The aim of this investigation was to study the cell biological and clinical aspects of the expression of the serine proteinase inhibitor skin-derived antileukoproteinase (SKALP) by human keratinocytes. Four main parts can be distinguished: part I (chapters 2 and 3) deals with the basic and molecular studies, part II (chapters 4 and 5) comprises the histological localization studies in normal and psoriatic tissue, part III (chapters 6 and 7) deals with the use of SKALP measurements in urine or serum for monitoring disease activity in psoriasis, and part IV (chapters 8 and 9) with the histological studies in human epidermal tumors.

INTRODUCTION

Polymorphonuclear leukocytes and proteinases

Polymorphonuclear leukocytes (PMN) are inflammatory cells with the potential to destroy tissues due to reactive oxygen metabolites and proteolytic enzymes. This destructive potential may be used beneficially as a defense mechanism against the environment, i.e. bacterial and fungal infection, but it may also be directed against the organism itself. PMN are known to cause tissue damage in many diseases [1]: arthritis [2-4], glomerulonephritis [5,6], cutaneous vasculitis [7], emphysema [8,9], adult respiratory distress syndrome [10,11], the allergic late phase asthmatic response [12], sepsis [13,14], bullous dermatoses [15-17], and tumor invasion and metastasis [18]. Most of the damage results from proteinases that degrade proteins in physiological and pathological processes [19,20]. PMN are known to contain several proteinases such as elastase, cathepsin G, collagenase, plasminogen activator, and proteinase 3.

The major PMN-derived proteinase in humans is human leukocyte elastase (E.C. 3.4.21.37), a serine proteinase with a broad range of substrates such as elastin, proteoglycans, collagen, fibrinogen, fibronectin, and keratins [8,21,22]. PMN contain 3 pg elastase per cell [23], which results in a daily turnover of more than 250 mg elastase [24], which has to be counteracted to prevent damage to normal tissue. Other elastases or elastase-like enzymes have been described in different cell types such as monocytes, eosinophils, basophils and mast cells [25-27].

Proteinase 3 has a substrate specificity similar to that of human leukocyte elastase [28]; it can induce emphysema in hamsters [29], and was shown to be the target antigen of anti-neutrophil cytoplasm auto-antibodies associated with Wegener's granulomatosis [30]. Furthermore, it may have a microbicidal action based on a nonproteolytic mechanism [31,32].

Proteinase inhibitors

Because of the nature of the function of the proteinases, a balanced homeostatic control of their activities is needed, and three principal *in vivo* mechanisms that prevent unwanted proteolytic tissue damage are known [21,33,34]. First, compartmentalization of enzymes by membranes provides a way to store injurious agents and prevents damage of tissues. Second, enzymes may be secreted in latent, inactive forms that require activation to degrade proteins, as is the case for metalloproteinases, and most of the serine proteinases and cysteine proteinases. Third, and relevant for this thesis, proteinase inhibitors are present as regulatory elements that counteract proteolytic activity by forming an inactive complex with their target proteinases.

The epidermis is an avascular tissue compartment to which nutrients and plasma proteins are supplied via diffusion from the dermal papillary vessels. During inflammation plasma protein levels are rapidly increased in epidermis [35], and diffusion of proteinase inhibitors together with other plasma components to the tissue site may occur [36]. However, in normal epidermis hardly any anti-elastase activity can be found [37], although the presence of α1-proteinase inhibitor (α1-PI) and α2-macroglobulin has been noticed in small quantities in normal skin [38,39].

Inflammation can be defined as the non-specific response of tissue following injury in order to restore homeostasis and tissue integrity. A destructive phase can be distinguished during which the stimulus is counteracted, followed by a phase of repair of the tissue in which hyperproliferation plays a role. Inflammatory mediators that are locally produced diffuse into the circulation and initiate a systemic reaction: the acute phase response [40]. This reaction includes fever, activation of the clotting system, and a change of cytokine production, which subsequently leads to the activation of inflammatory cells such as PMN, and an increased hepatic production of proteinase inhibitors [41]. This increased production of proteinase inhibitors such as α1-PI and α2-macroglobulin is thought to anticipate and prevent excessive proteolysis by PMN/monocyte-derived proteinases such as elastase, proteinase 3,

and cathepsin G. Some of these proteinase inhibitors may be present systemically, such as α1-PI, α1-antichymotrypsin, inter-α-trypsin inhibitor, and α2-macroglobulin, which are abundantly present in human blood plasma [33,42,43]. Inhibitors may also be produced locally such as SKALP (as is described in this thesis), the plasminogen activator inhibitors PAI-1 and PAI-2 [44], and ALP (antileukoprotease) [45,46], also known as SLPI (secretory leukocyte proteinase inhibitor) [47], as HUSI-I (human seminal plasma inhibitor) [48], as BSI-TE (bronchial secretion inhibitor with activity against trypsin, chymotrypsin, elastase and cathepsin G) [49], as BrPI (bronchial proteinase inhibitor) [50], and as MPI (mucous proteinase inhibitor) [51].

Inflammatory skin diseases

The cutaneous inflammatory response to injury, either trauma-induced or in skin diseases such as psoriasis or eczema, comprises mediators that are partly derived from activated keratinocytes [52]. These activated keratinocytes may produce cytokines that cause directional migration of leukocytes towards the site of inflammation [53] and activation of leukocyte function *in situ* [54]. This may result in a mixed accumulation of inflammatory cells such as macrophages, lymphocytes, and neutrophils [55]. Subsequently, the activated neutrophils and macrophages degranulate, and proteinases such as elastase and cathepsin G are released into the environment, i.e. the inflammatory site [56].

In normal skin, proteinases play an important physiological role in the process of keratinization by catalyzing the breakdown of nuclei and cell organells during transition from granular cells to horny cells. However, proteinases are also responsible for blister formation and acantholysis in pathological conditions such as several bullous diseases [16,44]. Naito et al reported that bullous pemphigoid blister formation was caused by PMN-proteinases, which were released after complement activation with production of chemotactic peptides that initiated PMN migration to basement membranes [57]. In patients with dermatitis herpetiformis high levels of elastase activity were present, probably derived from PMN that were abundantly present in the lesions [15]. *In vitro* experiments

demonstrated involvement of PMN elastase in the degradation of the basement membrane [58,59], and also *in vivo* the basement membrane appeared to be the target for human leukocyte elastase [60]. Eczema and psoriasis are other skin diseases in which PMN elastase is implicated [61-63]. *In vitro*, human leukocyte elastase appeared to impair epidermal intercellular cohesion, resembling *in vivo* findings in eczema such as spongiosis and acantholysis [64].

Epithelial cells produce not only plasminogen activators (urokinase and tissue plasminogen activator) under physiological conditions, but also the plasminogen inhibitors 1 and 2, that balance the activity of these proteolytic enzymes [44]. In psoriasis plasminogen activators are increased, especially the tissue type, which may activate the complement cascade and induce inflammation [65-67]. Furthermore, plasminogen activators appear to be associated with cell migration [68,69] and cell division [66,70], and may be involved in the increased rate of proliferation of the epidermal cells in psoriasis [71]. At inflammatory loci, proteinases may leak into surrounding normal tissue, and proteins need to be protected against destruction.

The serine proteinase inhibitors α1-PI and α1-antichymotrypsin are acute phase proteins that increase during inflammation, and that may fulfill this protective function [41].

Psoriasis

As mentioned above, PMN play a role in many diseases, and psoriasis is a skin disorder in which PMN invasion of the epidermis is an important and early feature [72,73]. Psoriasis is a common skin disease in the Western world, affecting about 2% of the people, men and women alike. Patients have sharply demarcated, erythematous lesions with a silvery white scaling, which may be minimal in size (pinpoint lesions), may be plaque-like, or may fuse and cover large areas of the skin [74]. A wide spectrum of involved area of the skin is seen, from solitary lesions that are restricted in size, to the most extensive form with (nearly) whole body involvement (erythroderma). Pustular forms are seen as well, both localized (palmoplantar pustulosis) or generalized (von Zumbusch type).

In addition, extracutaneous manifestations of psoriasis affecting nails, mucosal membranes, and joints may be present [75].

Histologically, psoriasis is characterized by inflammatory changes in dermis and epidermis such as dilation of the capillaries in the papillary dermis with extravasation of lymphocytes, erythrocytes, macrophages, and PMN; edema of the papillary dermis, hyperkeratosis, parakeratosis, disappearance of the granular layer, spongiosis and pustule formation in the epidermis are seen as well. Reports on the sequence of the events conflict [76-80]. Most groups agree that dermal inflammatory events precede epidermal changes, although altered keratinocyte differentiation preceding vascular abnormalities during the spread of psoriatic plaques has been described [76]. PMN involvement is regarded as an important feature of psoriasis [63,73]. While both in the early phase of the psoriatic lesion and in the pustular forms of psoriasis PMN invasion of the epidermis is the histologically predominant event [72,74], in chronic stable plaque psoriasis the intra-epidermal PMN accumulation is less impressive [81].

Skin-derived antileukoproteinase (SKALP): review of the data available at the start of the project

In 1988, elastase-inhibiting activity could be demonstrated in lesional psoriatic epidermis and scales of lesional epidermis, whereas extracts from normal human skin showed hardly any inhibition of elastase [82-84]. The inhibiting activity was caused by a low molecular weight endogeneous inhibitor that was heat stable and resistant to extremes of pH. Furthermore, the inhibitor could be induced by experimental inflammation. Psoriatic scales were used as a source to purify the inhibitor for characterization experiments. The inhibiting activity appeared to be caused by two species of new inhibitors with molecular weights of 10 and 20 kDa, as was shown on SDS-PAGE and by gel permeation experiments [37]. The inhibitors expressed a strong specific inhibition towards human leukocyte elastase with a $K_i < 10^{-10}$ M, since there was no interference with cathepsin G, which is inhibited by other elastase inhibitors. Sellotape stripping as an *in vivo* model for wound

healing, yielding an inflammatory reaction of the skin and hyperproliferation of keratinocytes [85], induced elastase-inhibiting activity [86]. In view of the kinetics with maximal inhibiting activity 48 hours after injury, it was suggested that the inhibitor might function as an off-switch of epidermal inflammation [83]. Because amino acid sequencing demonstrated partial homology with the elastase inhibitor antileukoprotease (ALP) this new elastase inhibitor was named skin-derived antileukoproteinase (SKALP).

Two inhibitors derived from bronchial secretions were similar to SKALP with respect to inhibition of human leukocyte elastase, but were different with respect to the interference with trypsin, chymotrypsin or cathepsin G. Hochstrasser et al reported a low molecular weight inhibitor specific to human leukocyte elastase and porcine pancreatic elastase, but with other inhibiting characteristics ($K_i=5\cdot10^{-10}$M for HLE) and another iso-electric point (pI>11) than SKALP [83,87]. Rasche et al [88] described the same elastase-specific inhibitor in human bronchial mucus (BSI-E), which might be present in a masked form, probably in complex with proteases. The rapid oxidative inactivation of the inhibitor made it unlikely that this molecule was identical to SKALP. Kramps et al described an elastase-specific inhibitor from human bronchial secretion, which was named LMI-5000 because of its low molecular weight of 5000 Da. The lower molecular weight, the elastase-inhibiting activity under reducing conditions, and the amino acid composition were different from the known properties of SKALP [89]. However, our own antisera raised against SKALP isolated from psoriatic scales did react with this LMI-5000.

The elastase-inhibiting activity was demonstrated not only in psoriasis and after sellotape stripping, but also in other scaling skin disorders [90]. In the erythrodermic form of autosomal recessive lamellar ichthyosis and in Netherton syndrome, elastase-inhibiting activity was significantly increased. These monogenic disorders of keratinization are also accompanied by inflammation. Non-inflammatory monogenic disorders of keratinization and atopic dermatitis showed only a slight increase in elastase-inhibiting activity as compared to normal skin [90].

In the spreading psoriatic lesion SKALP activity was maximal in the center and decreased towards the marginal zone as measured by a functional assay; more than 4 mm outside the edge of the lesion SKALP activity was similar to that in normal skin [91]. Intra-epidermal PMN accumulation is increasing from the center of the psoriatic lesion towards the periphery where psoriasis is active [91,92].

SKALP was further characterized and appeared to be a very cationic molecule, which could be present in an intact form or in a degraded form with a lower molecular weight [93]. The intact molecule with a molecular weight of approximately 18 kDa was the main form in cultured keratinocytes, whereas the 11 kDa fragment was the predominant form in psoriatic scales. The latter was subjected to N-terminal gas-phase sequencing, and within a stretch of 16 amino acids, a 40% homology was found with the C-terminal half of ALP (comprising the reactive site). Apart from human leukocyte elastase, porcine pancreatic elastase (PPE) was also inhibited, and the iso-electric point appeared to be pI>10.5 [93].

At the same time, elafin, an elastase-specific inhibitor of human skin was described by Wiedow et al [94]. Complete N-terminal sequencing showed that the inhibitor was composed of 57 amino acids, and a molecular weight of 7017 Da was reported. Wiedow et al performed experiments that proved elafin not only to inhibit human leukocyte elastase and porcine pancreatic elastase, but also proteinase 3, an elastin-degrading proteinase from neutrophils [95].

The presence of an elastase-specific inhibitor in lung tissue as described by Hochstrasser et al, Rasche et al, and Kramps and Klasen [87-89], was confirmed and described in greater detail by Sallenave et al. They reported an elastase-specific inhibitor with a molecular weight of 2.5 kDa [96], which was very similar to a portion of elafin and which showed some homology with ALP, especially around the reactive site fo human leukocyte elastase.

Aim of this project

At the start of the project only biochemical data were available. Two related projects were set up for the molecular and functional characterization of SKALP: the project described here, and the project 900-512-146 of the 'Netherlands Organization for Scientific Research (NWO)' which deals with the molecular structure and regulation of the expression of SKALP. The aims of the project described in this thesis were:

1. To study cell biological aspects of SKALP such as distribution and localization *in vivo*, and the expression by cultured keratinocytes.
2. To investigate the clinical applications of SKALP measurement in human tissues.

Specific questions of this project were:

1. Which cells are responsible for the production of SKALP in skin?
2. Is SKALP expression restricted to skin, or is SKALP also produced in other issues?
3. Which factors induce SKALP expression *in vivo* and *in vitro*?
4. What is the expression pattern in skin diseases?
5. Can SKALP be used as a marker for disease activity in psoriasis?

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Chapter 2

SKALP/elafin: an elastase inhibitor from cultured human keratinocytes. Purification, cDNA sequence, and evidence for transglutaminase cross-linking

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ABSTRACT

SKALP/Elafin is a proteinase inhibitor found in psoriatic epidermis as a short polypeptide of 6 kDa. Here we present evidence that this protein is synthesized as a larger precursor molecule with distinct biological features. Purification and NH_2-terminal sequencing of SKALP/elafin from cultured human keratinocytes and the cloning of its cDNA revealed the existence of a mature protein, which upon cleavage of a hydrophobic signal sequence of 22 amino acids has a calculated molecular mass of 9.9 kDa (95 amino acids). In addition to the known proteinase inhibitor domain, the mature protein contains a domain with 4 repeats which are homologous to putative transglutaminase substrate motifs. We were able to demonstrate on Western blots that immunoreactive SKALP is present in high molecular weight proteins extracted from psoriatic skin. This suggests that SKALP is covalently attached to epidermal proteins. In addition it was found that both the complete SKALP molecule and a synthetic peptide of the NH_2-terminal portion of SKALP could be used as a transglutaminase substrate. We therefore speculate that SKALP/elafin, secreted by epidermal keratinocytes in inflamed skin, exists both as a free 6-kDa form and as an immobilized 9.9-kDa form covalently attached to the cornified envelopes by transglutaminase cross-linking.

INTRODUCTION

Elastase is a serine proteinase derived from polymorphonuclear leukocytes [1]. When secreted at sites of inflammation this enzyme can cause severe tissue damage, since it is active at neutral pH and can degrade a broad range of connective tissue components such as elastin [2], proteoglycans [3] and collagen [4,5]. The action of elastase, like that of many other proteinases, is controlled by proteinase inhibitors which are found in the extracellular fluids [6]. A crucial role for proteinases and their inhibitors has been suggested for several pathologic conditions,

such as emphysema [2,7], arthritis [3,8], nephritis [9], and certain skin diseases [10,11].

Recently we have described a new, low molecular weight elastase inhibitor derived from psoriatic skin [12,13] termed SKALP (skin-derived antileukoproteinase). We demonstrated that this inhibitor can be found in the epidermis of several inflammatory skin diseases, but not in normal human epidermis. SKALP, also termed elafin by Wiedow *et al.* [14], was found both in the epidermis and in the urine of psoriatic patients [15]. Immunohistochemical studies showed that SKALP/elafin is found in the suprabasal differentiated keratinocytes of psoriatic epidermis [16].

In previous studies [12,13] we have shown that cultured human keratinocytes contain an elastase inhibitor with an apparent molecular mass of 18 kDa on SDS-PAGE, which is otherwise indistinguishable from SKALP/elafin derived from psoriatic epidermis using biochemical and immunochemical criteria. Here we describe the purification, NH_2-terminal sequencing, and cDNA sequence analysis of this elastase inhibitor from cultured keratinocytes. We conclude that this protein with a molecular mass of 9.9 kDa represents the precursor molecule of the 6-kDa form of SKALP/elafin found in psoriatic epidermis. The NH2-terminal part of this precursor was also shown to contain a domain with putative transglutaminase substrate motifs. Here we present evidence that transglutaminase-mediated cross-linking of SKALP can occur in human epidermis *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Keratinocyte culture and extraction

Human epidermal keratinocytes were cultured according to the Rheinwald-Green system [17] as previously described [18]. Cells were grown to confluence and lysed in 20 ml 0.025 mM triethylamine per 100-ml tissue culture flask, containing approximately 20 x 10^6 cells. Cell extracts were prepared by sonication on ice followed by centrifugation for 1 h at 40,000 x *g*. The supernatant was concentrated by vacuum

centrifugation in a Speed-Vac concentrator (AS160, Savant). The sample was dialyzed against distilled water, again centrifuged, and used for chromatography. Using this procedure, 100-200 ng of SKALP was obtained per 10^6 cells, assuming that all antielastase activity measured in the crude extract represents SKALP with a molecular mass of 9.9 kDa.

Biopsies

Human skin biopsies (0.3 mm thickness) from healthy volunteers and psoriatic patients were taken with a keratotome as previously described [12] and used for keratinocyte cultures and RNA extraction.

Purification of SKALP

Extracts from cultured keratinocytes were either directly used for reversed-phase chromatography or first purified on a PBE 118 chromatofocusing column as described before [13]. Samples were chromatographed on a µRPC C2/C18 PC 3.2/3 reversed-phase column using the SMART chromatography system (Pharmacia LKB Biotechnology INC.) at a flow of 240 µl per min. The gradient was 10 min of 100% buffer A (0.1% trifluoroacetic acid in distilled water), then 100% buffer A to 70% buffer B (0.1% trifluoroacetic acid in acetonitril) in 30 min (linear gradient) followed by a 5-min gradient to 100% buffer B. UV absorption was continuously recorded at 215 nm. The anti-elastase activity of all fractions was measured fluorimetrically using a synthetic substrate as described before [13], and active fractions of several runs were pooled and rechromatographed using the same conditions. The fraction containing peak activity was freeze-dried, dissolved in phosphate-buffered saline, and subjected to gel permeation chromatography on a Superdex 75 PC 3.2/30 column (Pharmacia) using the SMART chromatography system. The column was equilibrated with phosphate-buffered saline, and a flow rate of 80 µl per min was applied. The column was calibrated with Blue dextran (void volume), ovalbumin (43 kDa), cytochrome c (12 kDa), and L-dopa (total volume) as molecular mass markers (Sigma). Fractions were collected and antielastase activity was found in the major peak eluting from the

column. This material was analyzed (Eurosequence Inc., Groningen, The Netherlands) on a gas-phase sequenator (Model 477A) and an on-line HPLC (Model 120A), from Applied Biosystems. The material was not alkylated or otherwise modified prior to sequencing.

Synthesis of a specific cDNA hybridization probe

Total RNA was isolated from psoriatic epidermis by the single-step guanidinium thiocyanate method of Chomczynski and Sacchi [19]. First strand cDNA was generated from total RNA with Moloney murine leukemia virus RNase H- reverse transcriptase (GIBCO-BRL Life Technologies) under conditions as suggested by the supplier, using excess oligo(dT) primers. Part of the total reverse transcriptase (RT) reaction was used for mixed oligonucleotide-primed amplification of cDNA [20] to generate a SKALP-specific hybridization probe. Based on amino acid sequence data of elafin [14] degenerate PCR-primers were synthesized on a Gene Assembler Plus (Pharmacia). As the sense primer the degenerate codons for amino acids 1 to 7 were taken. The antisense primer represents amino acids 40 to 46, and the internal antisense primer contains the possible sequences encoding amino acids 33 to 39 in elafin (see also Fig. 2). All primers contained an additional *Bam*HI site to facilitate subcloning of the amplified DNA. PCR-reactions were carried out using a DNA thermal Cycler (Perkin Elmer/Cetus) in 50-µl mixtures covered with mineral oil. The following buffer conditions were used: 16.5 mM ammonium sulfate, 65 mM Tris-HCl, pH 8.8, 0.065 mM EDTA, 0.17 mg/ml bovine serum albumin, 10 mM β-mercaptoethanol, 3 mM magnesium chloride, 0.2 mM of each dNTP and 500 ng of each primer. After an initial boiling of 10 min the temperature was lowered to 80°C, and 1 unit of Taq polymerase was added. Amplification was for 30 cycles as follows: 60 s at 94°C, 90 s at 42°C, 90 s at 72°C. An additional 10 min at 72°C was used for the last cycle. To determine the specificity of the resulting PCR product, the DNA was purified from agarose gel and used as a template in a second PCR reaction under the same conditions with the sense primer and the internal antisense primer. To rescue the original amplified cDNA, purified cDNA was treated with

Proteinase K [21], digested with *Bam*HI, subcloned into the *Bam*HI-sites of a pGEM-4 vector (Promega) and sequenced using the dideoxy chain termination procedure [22].

Library screening

To determine the hybridization conditions for library screening with a 243-base pair PCR-generated cDNA, this cDNA was initially used as a probe for Southern blot analysis of human blood cell genomic DNA following standard procedures [23]. Based on the results of this analysis, a random primed human keratinocyte cDNA library in the vector λgt11 (HL1110b, Clonetech) was screened with replica filters (Hybond N+, Amersham Corp.) at 65°C in hybridization buffer (500 mM sodium, 250 mM phosphate, pH 7.2, 7% SDS, 1 mM EDTA). Washing of the filters was performed at 65°C in wash buffer (250 mM sodium, 125 mM phosphate, 1% SDS, 1 mM EDTA). The probe was labeled with ^{32}P by random priming following standard procedures [23]. Hybridizing phage plaques were purified and λDNA was isolated. A *Sac*I/*Kpn*I-insert from one positive λ clone was subcloned into pGEM-4 and used for further analysis. Based on the results of Southern blot analysis of this clone, using the 243-base pair PCR-generated cDNA as a probe, an 0.5-kilobase pair *Eco*RI fragment was subcloned into pGEM-4, and both strands were completely sequenced.

Computer analysis

DNA sequence gel readings were recorded and edited using the IG-suite 5.35 package (Intelligenetics, Inc.) on a SUN computer. Protein analysis was carried out using the CAMMSA software package on a VAX computer as provided by the Dutch CAOS/CAMM center.

Antisera

Antisera were raised against the 6-kDa form of SKALP/elafin which lacks the transglutaminase substrate domain. We used both recombinant elafin (a kind gift of Dr. N. Russell, ICI, United Kingdom) or SKALP purified from psoriatic scales [13]. Protein (50 μg) was cross-linked with

glutaraldehyde, dialysed against distilled water and emulsified with Freund's complete adjuvant [13]. Rabbits were immunized intracutaneously in the back, followed by two subcutaneous boosters at week 2 and 4. Preimmune serum was drawn before the experiments and immune serum was collected after 6 weeks. Titers in enzyme-linked immunosorbent assay were 1/20000. On Western blots, dilutions between 1/100 and 1/1000 were used. The antisera were negative with extracts of normal human skin and human plasma proteins. Antisera raised against recombinant material and against purified protein from scales yielded identical staining patterns.

SDS-PAGE and Western blotting

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electroforesis (SDS-PAGE) on a 16 % gel, using tricine as a trailing ion instead of Tris-glycine [24], or on a 12% gel using the conventional SDS-PAGE [25]. Molecular mass markers used were: broad range prestained markers from Bio-rad (myosin (205 kDa), β-galactosidase (116 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa), lysozyme (18.5 kDa), and aprotinin (6.5 kDa)), and ^{14}C-methylated proteins from Amersham (myosin (200 kDa), phsophorylase b (92 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa)). Molecular masses according to the manufacturers. Gels were electroblotted on polyvinylidenedifluoride (PVDF) membranes (Millipore), and protein staining was performed with Amido Black according to standard procedures. Immunological detection of proteins was performed using the antisera described above, followed by biotinylated swine-anti-rabbit Ig and avidin-conjugated alkaline phosphatase using a colorimetric detection system [15]. Preimmune serum of the same rabbits was used as a control.

Preparation of a biotinylated peptide

A peptide comprising amino acids 23 to 36 of SKALP (see also Fig. 2),

containing a glutamine which is part of a GQDXVK motif, putatively involved in cross-linking, was synthesized (Eurosequence Inc, Groningen, The Netherlands). The peptide (250 μg) was biotinylated with 500 μg of NHS-LC-biotin (Pierce) for 2 h at 37°C in 300 μl of 0.1 M Na_2CO_3, pH 8.0. The reaction was stopped by the addition of 20 μl of 1 M Tris, pH 8.0 [26].

Cross-linking of the biotinylated peptide to stratum corneum proteins by endogenous and exogenous tranglutaminase

Scales from a psoriatic patient (1 gram) were homogenized in 20 ml of phosphate buffered saline and centrifuged for 30 min at 40,000 x *g*. The supernatant (2 mg of protein per ml) was sterilized through a 0.2-μm filter and stored at -20°C until further use. For cross-linking experiments 10 μl of scale extract was used with 8 μl of biotinylated peptide solution [26]; either 0.01 unit of guinea pig liver transglutaminase (Sigma) or water (to measure the endogenous transglutaminase activity of the scale extract) was added. The reaction was initiated by the addition of $CaCl_2$ at a concentration of 8.7 mM, in a final reaction volume of 20 μl. A reaction mixture containing 50 mM of EDTA was used as a control. After 16 h at 37°C the reaction mixtures were diluted with SDS-sample buffer (containing dithiothreitol) and boiled for 2 min. Samples were subjected to SDS-PAGE (12% gel) and blotted onto PVDF membrane. Biotinylated proteins were detected with the Western Light kit (Tropix) according to the manufacturer's instructions. This assay uses avidin-conjugated alkaline phosphatase and chemiluminescence of a sensitive alkaline phosphatase substrate. Positive bands were printed on x-ray film.

Labeling of purified SKALP with [^{14}C]methylamine; detection of ^{14}C-labeled protein

SKALP purified from cultured keratinocytes as described above, was assayed to act as an amine acceptor using [^{14}C]-methylamine as a probe [27]. SKALP (1 μg) was dissolved in 50 mM Tris, 100 mM NaCl, pH 7.5, containing 20% glycerol, 2 mM leupeptin, 1 mM

phenylmethanesulfonylfluoride, 8.7 mM $CaCl_2$, 0.01 unit guinea pig liver transglutaminase, and 1 MBq of [^{14}C]methylamine (specific activity, 2.04 GBq per mmol) in a final volume of 25 µl. After 2 h at 37°C the reaction was terminated by the addition of EDTA (20 mM final concentration). From this mixture 250 ng of SKALP was run on SDS-PAGE (16% gel) and blotted onto PVDF membrane as described above. ^{14}C-Labeled proteins (Amersham) were used as molecular mass markers. The dried blot was used for autoradiography on x-ray film, with two intensifying screens, for 62 h.

RESULTS

Purification and NH_2-terminal sequencing of SKALP

Reversed-phase chromatography of soluble proteins extracted from cultured keratinocytes yielded one peak of antielastase activity as shown in Fig. 1a. Pooled fractions were, after rechromatography, finally purified on a Superdex 75 gel permeation column which gave a peak of antielastase activity coinciding with the major peak recorded at 215 nm (Fig. 1b). A K_{av} of 0.28 corresponding with an apparent molecular mass of 22 kDa was calculated. NH_2-terminal gas-phase sequencing of this material identified 26 amino acids (see Fig. 2) of which the 7 COOH-terminal amino acids overlap with the NH_2-terminal sequence of the 76-amino acid form of elafin purified from psoriatic scales [28]. In Fig. 2 the amino acid sequence of SKALP from cultured keratinocytes is compared with the known sequence data of elafin from psoriatic scales.

cDNA probe synthesis, library screening, and sequence analysis

In order to verify and extend the protein sequence data we cloned and sequenced the SKALP-encoding cDNA. Amplification of cDNA reverse-transcribed from psoriatic epidermis total RNA with the sense and antisense primer (Fig. 2) resulted in 2 distinct cDNAs. The largest cDNA of about 250 base pairs was used for a second PCR reaction with the sense primer and the internal antisense primer (Fig. 2). This resulted in a shorter PCR product of about 230 base pairs. The 250-base pair product

from the initial PCR reaction was subsequently subcloned into the *Bam*HI site of a pGEM-4 vector.

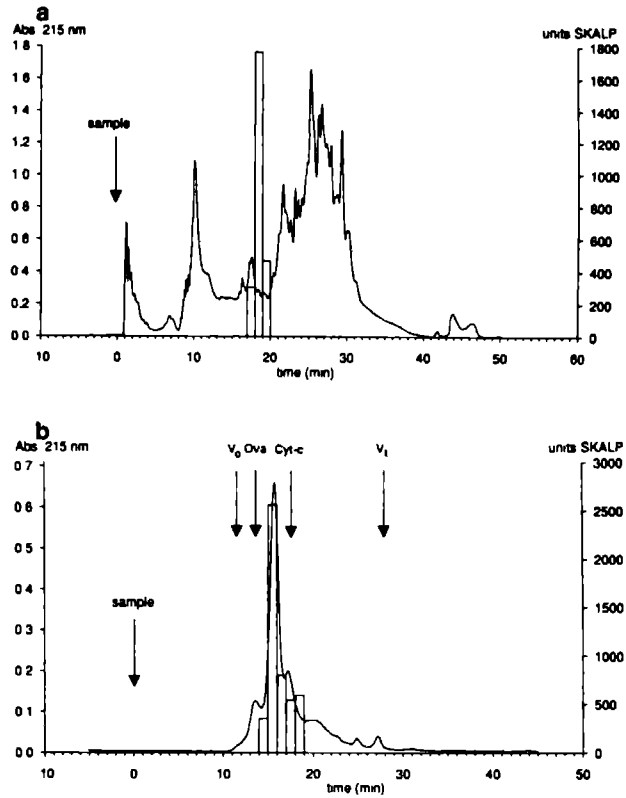

Fig. 1. *a*, reversed-phase chromatography (μRPC C2/C18) of soluble proteins from cultured keratinocytes. *Solid line*, absorbance at 215 nm. *Bars*, antielastase activity expressed as units per fraction. 1 unit is the amount of inhibitor that gives 50% inhibition of 1 nmol of human leukocyte elastase [13]. *b*, gel permeation chromatography (Superdex 75) of partially purified SKALP obtained by reversed-phase chromatography. *Solid line*, absorbance at 215 nm. One fraction per minute was collected. *Bars*, antielastase activity in units per fraction. The material from fraction 16 was used for NH_2-terminal sequencing. V_O, void volume; *Ova*, ovalbumin (43 kDa); *Cyt-c*, cytochrome c (12 kDa); V_t, total volume.

One subclone, pGSKA1, containing the desired insert was characterized by DNA sequence analysis. The predicted amino acid sequence of the

insert of clone pGSKA1 corresponded to the region from amino acid 31 to 107 in Fig. 2.

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                                                          5'-CC  002

Met Arg Ala Ser Ser Phe Leu Ile Val Val Val Phe Leu Ile Ala  015
ATG AGG GCC AGC AGC TTC TTG ATC GTG GTG GTG TTC CTC ATC GCT  047
                            ▼
Gly Thr Leu Val Leu Glu Ala Ala Val Thr Gly Val Pro Val Lys  030
GGG ACG CTG GTT CTA GAG GCA GCT GTC ACG GGA GTT CCT GTT AAA  092

Gly Gln Asp Thr Val Lys Gly Arg Val Pro Phe Asn Gly Gln Asp  045
GGT CAA GAC ACT GTC AAA GGC CGT GTT CCA TTC AAT GGA CAA GAT  137

Pro Val Lys Gly Gln Val Ser Val Lys Gly Gln Asp Lys Val Lys  060
CCC GTT AAA GGA CAA GTT TCA GTT AAA GGT CAA GAT AAA GTC AAA  182

Ala Gln Glu Pro Val Lys Gly Pro Val Ser Thr Lys Pro Gly Ser  075
GCG CAA GAG CCA GTC AAA GGT CCA GTC TCC ACT AAG CCT GGC TCC  227

Cys Pro Ile Ile Leu Ile Arg Cys Ala Met Leu Asn Pro Pro Asn  090
TGC CCC ATT ATC TTG ATC CGG TGC GCC ATG TTG AAT CCC CCT AAC  272

Arg Cys Leu Lys Asp Thr Asp Cys Pro Gly Ile Lys Lys Cys Cys  105
CGC TGC TTG AAA GAT ACT GAC TGC CCA GGA ATC AAG AAG TGC TGT  317

Glu Gly Ser Cys Gly Met Ala Cys Phe Val Pro Gln END          117
GAA GGC TCT TGC GGG ATG GCC TGT TTC GTT CCC CAG TGA GAGGGAG  363

CCGGTCCTTGCTGCACCTGTGCCGTCCCCAGAGCTACAGGCCCCATCTGGTCCTAAGTC  422

CCTGCTGCCCTTCCCCTTCCCACACTGTCCATTCTTCCTCCCATTCAGGATGCCCA-3'  478
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Fig. 2. Nucleotide sequence and amino acid sequence of preSKALP. The first 18 base pairs of the cDNA are not indicated in this figure (see "Results"). The deduced amino acid sequence reveals a protein of 117 amino acids. The numbering of the amino acids starts with the methionine. The cleavage site for the putative signal sequence (▾) is indicated. Amino acids 23 to 48 and amino acids 85 to 100 were confirmed by NH_2-terminal gas-phase sequencing of SKALP from cultured human keratinocytes (see "Results") and from psoriatic scales [13], respectively. Amino acids 61 to 117 and amino acids 42 to 60 (*underlined*) are identical to, respectively, the amino acid sequence of elafin [14] and an additional NH_2-terminal amino acid sequence of elafin [28]. The sites to which the PCR primers were directed are *double underlined.*

Subsequent screening of a human keratinocyte cDNA library ($1x10^5$ clones) with pGSKA1 as a probe yielded three positive clones with larger inserts. After plaque purification and rescreening, the largest, clone F1Ac53, was selected for further analysis. An *Eco*RI-fragment which strongly hybridized with the cDNA probe, was subsequently subcloned into pGEM-4. Sequence analysis of the resulting subclone,

designated pGESKA, revealed an insert comprising 496 base pairs, including an open reading frame coding for a 117-amino acid protein (Fig. 2) with a calculated molecular mass of 12.3 kDa, an isoelectric point of 8.84 and a putative signal sequence of 22 amino acids [29]. The first 18 base pairs of the 5'-untranslated region are scrambled with a 28 S rRNA gene fragment. The 3'-untranslated region comprises 122 nucleotides and does not contain a poly(A)-tail nor a polyadenylation signal since a random primed cDNA library was used.

Cross-linking of SKALP

The NH_2-terminal part of the 9.9-kDa SKALP-protein (amino acid 23 to 68 in Fig. 2) from cultured keratinocytes contains four repeats which match remarkably well with a consensus sequence for transglutaminase cross-linking, first described in seminal vesicle protein I from guinea pig [30,31]. Covalent clotting of this protein is catalyzed by a transglutaminase, involving the formation of γ-glutamyl-ε-lysine cross-links. Three residues in these repeats, 2 lysines and 1 glutamine, are thought to participate in the cross-links. Fig. 3 shows the repeats in SKALP as compared to the supposed consensus sequence for transglutaminase-catalyzed cross-linking.

Consensus: GQDxVKxxxxxK

Repeat a: 31GQDTVKGRVPFN42
Repeat b: 43GQDPVKGQVSVK54
Repeat c: 49GQVSVKGQDKVK60
Repeat d: 55GQDKVKAQEPVK66

Fig. 3. Comparison of four repeats in the putative transglutaminase substrate domain with the supposed consensus sequence for transglutaminase cross-linking [30,31]. Identical amino acids are marked. Numbering is according to Fig. 2.

To investigate whether SKALP can act as a transglutaminase substrate, three strategies were followed.

First, we extracted proteins from psoriatic scales directly in SDS-sample buffer and ran them on SDS-PAGE (16% gel). The proteins were blotted on PVDF membrane and stained with an antiserum against the low molecular weight (6 kDa) form of SKALP. As shown in Fig. 4*a* (*lane 4*) several forms of SKALP appear to be present in scale extract (including the 9.9- and the 6-kDa forms). It should be noted that the molecular mass determination of SKALP is not reliable on these gels (as indicated by the marker proteins) due to the cationic nature of the molecule as described before [13,14].

In addition to the expected SKALP forms, in *lane 4*, positive bands in the higher molecular weight range are visible, suggesting that SKALP is covalently attached to other proteins. No positive reaction of the antisera with high molecular weight proteins in extracts of normal skin was found. For comparison partially purified SKALP/elafin from psoriatic scales (*lane 1*), purified SKALP from cultured cells (*lane 3*) and recombinant elafin/SKALP (*lane 2*) were stained on the same blot as shown in Fig. 4*a*.

Second, we used a biotinylated synthetic peptide comprising amino acids 23 to 36 of SKALP, as a substrate for transglutaminase. The peptide was derived from the NH_2-terminal domain and contained a glutamine of a putative transglutaminase substrate motif as a potential amine acceptor. The biotinylated peptide was added to extracts of psoriatic scales, with or without exogenous transglutaminase. The presence of an excess EDTA was used as a control for the Ca^{2+}-dependent reaction. Fig. 4*b* shows that the biotinylated peptide is cross-linked to many skin proteins in the absence of exogenous transglutaminase (*lane 2*), presumably by the action of endogenous epidermal transglutaminase which is known to be present in psoriatic scales [32]. Addition of liver transglutaminase (*lane 3*) clearly increases the amount of labeled proteins. EDTA almost completely blocks the formation of biotinylated skin proteins (*lane 4*), presumably by inhibiting transglutaminase activity.

Third, highly purified SKALP from cultured keratinocytes (9.9-kDa form) was used as a substrate for guinea pig liver transglutaminase,

using [^{14}C]methylamine as an acyl-acceptor probe. Fig. 4*c* shows that SKALP can indeed act as a substrate in this reaction.

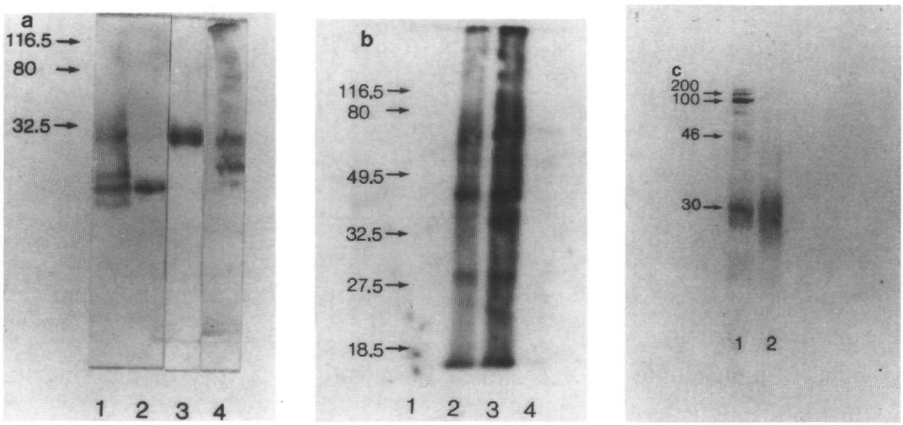

Fig. 4. *A*, immunoblots of SKALP preparations and scale extracts with anti-SKALP serum. *Lane 2*, recombinant elafin/SKALP (6 kDa); *lane 3*, purified SKALP from cultured keratinocytes (9.9 kDa); *lane 1*, partially purified SKALP/elafin from psoriatic scales, containing multiple forms of SKALP/elafin, including the 6- and 9.9-kDa forms; *lane 4*, total scale extract showing additional high molecular weight proteins, suggesting cross-linking of SKALP to proteins of the stratum corneum. *B*, autoradiograph from a Western blot of SDS-PAGE demonstrating linkage of a biotinylated peptide to stratum corneum proteins by transglutaminase. *Lane 1*, scale extract without biotinylated peptide; *lane 2*, scale extract with biotinylated peptide; *lane 3*, scale extract with biotinylated peptide and exogenous transglutaminase; *lane 4*, scale extract with biotinylated peptide in the presence of EDTA. *C*, autoradiograph from a Western blot of SDS-PAGE showing purified SKALP (9.9-kDa form) as an amine acceptor with [^{14}C]-methylamine as a probe (*lane 2*). Radiolabeled markers are shown in *lane 1*.

DISCUSSION

From comparison of the predicted amino acid sequence of our cloned cDNA with the known amino acid sequence of elafin and our protein sequence data of SKALP (Fig. 2) we conclude that clone pGESKA encodes the complete preSKALP protein.

When this manuscript was in preparation, the genomic organization and complete nucleotide sequence of the human elafin gene were reported [33]. Comparison of our cDNA with the predicted exon sequen-

ces shows 100% homology, confirming that SKALP and elafin indeed are identical proteins.

Three domains in preSKALP

A signal peptide cleavage site in preSKALP is predicted [29] between amino acid residues 22 and 23. Based on the genomic DNA sequence [33] it was speculated that the signal peptide spans a region of 25 amino acids. However, this was not supported by sequence data at the protein level. Our results from the NH_2-terminal gas-phase sequencing of SKALP purified from cultured keratinocytes clearly show that the protein starts at amino acid 23 from the cDNA-deduced amino acid sequence (Fig. 2). We therefore conclude that this protein indeed is the preSKALP polypeptide, and that the site of cleavage for the signal peptide as we predict it must be correct.

The presence of a putative signal sequence suggests that translation of SKALP-coding mRNA takes place at the rough endoplasmic reticulum [34]. It is not yet clear through which intracellular routes the protein is finally secreted, and this is currently under investigation.

The COOH-terminal part of SKALP contains 8 cysteine residues which are probably involved in disulfide bonds. Small proteins showing this characteristic have been called "four-disulfide core" proteins [35]. Several proteins have been shown to belong to this group, like whey acidic protein [36], the sodium potassium ATPase inhibitors 1, 2 and 3 [37] and antileukoprotease/secretory leukocyte protease inhibitor (ALP/SLPI) [38,39]. The COOH-terminal part of ALP/SLPI shows 40% homology with the COOH-terminal part of SKALP (amino acid 68 to 117 in Fig. 2) and especially the positions of the cysteine residues are highly conserved. The NH_2-terminal parts of SKALP and ALP/SLPI show no significant homology. Based on the homology between the COOH-terminal parts of SKALP and ALP/SLPI, we denote the domain spanning amino acid 68 to 117 (Fig. 2) of SKALP as being the proteinase inhibiting domain.

The NH_2-terminal part of SKALP was shown to contain four repeats, highly homologous to a consensus sequence for transglutaminase cross-linking, as we already mentioned (see "Results"). Based on this striking

homology, we surmised that this region can act as a putative substrate domain for epidermal transglutaminase. Our transglutaminase experiments clearly show that SKALP can indeed become cross-linked by transglutaminase to proteins extracted from psoriatic scales. It is therefore tempting to speculate that SKALP can exist as an immobilized 9.9-kDa protein, covalently attached to the cornified envelopes by transglutaminase cross-linking. Currently, experiments with the 9.9-kDa form of SKALP as a product of recombinant DNA technology, in combination with site-directed mutagenesis, are in progress in order to determine the exact sites used for cross-linking.

Immunohistochemical staining of psoriatic epidermis suggests that at least part of the SKALP present in the epidermis is indeed closely associated to the cornified envelopes [16]. However, extraction and purification of SKALP/elafin from psoriatic scales predominantly yields a low molecular weight form [13,14]. We therefore speculate that in psoriatic lesions a mechanism exists to cleave the proteinase inhibiting domain from the putative transglutaminase domain. Cross-linking to the cornified envelopes might be a prerequisite for this mechanism since, in cultured keratinocytes, where cornified envelopes are not present, we mainly find the complete 9.9-kDa protein. Further studies on *in vitro* and *in vivo* differentiation of keratinocytes should give us a more detailed insight into the pathways of SKALP processing.

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Chapter 3

SKALP/elafin is an inducible proteinase inhibitor in human epidermal keratinocytes

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ABSTRACT

Skin-derived antileukoproteinase (SKALP), otherwise known as elafin, is a recently discovered epidermal proteinase inhibitor with specificity for polymorphonuclear leukocyte (PMN)-derived elastase and proteinase-3; in addition to the proteinase-inhibiting domain, SKALP contains several transglutaminase substrate motifs. SKALP is virtually absent in normal human epidermis but is found in a number of inflammatory skin diseases, including psoriasis. Here we report the induction and processing of SKALP in vivo and in vitro. SKALP-expression in vivo could be demonstrated following injury in normal human epidermis, using histology, Western blotting, Northern blotting and a functional assay. In vitro, SKALP expression was studied in conventional submerged keratinocyte culture systems and in keratinocytes cultured in an air-liquid interface model. Induction of SKALP-activity in epidermis could be measured as early as 16 h after skin injury; immunohistological examination showed that SKALP expression was confined to the outer layers of the stratum spinosum and the stratum granulosum. Northern blot analysis revealed a 0.8 kb transcript, both in vivo (psoriatic skin, injured skin) and in vitro (cultured keratinocytes). Western blot analysis showed that the major SKALP form in vivo was a low molecular mass fragment, containing the antiproteinase domain. In all cultures that were positive for SKALP, larger (8-10 kDa) forms of SKALP, containing the N-terminal transglutaminase substrate motifs in addition to the antiproteinase domain, were found. SKALP expression in cultured cells was found to be dependent on the system used. In a submerged culture system, SKALP could be induced by fetal calf serum.

These findings demonstrate that SKALP is an inducible proteinase inhibitor and support the concept that SKALP acts as a regulatory molecule in cutaneous homeostasis.

INTRODUCTION

Human epidermis is a stratified keratinizing epithelium that follows a

highly coordinated process of differentiation leading to a layer of dead cells, and forming the protective covering of the skin (for reviews, see [1,2]). Under pathological conditions (e.g. psoriasis) or during regenerative and adaptive processes (e.g. wound healing), human epidermis switches from the normal differentiation program to a 'hyperproliferative' differentiation program. This includes the expression of different sets of cytokeratins [3], a premature expression of differentiation markers (involucrin, transglutaminase) in the spinous cell layers [4], and an increase of cycling cells, as measured by the expression of Ki-67 antigen [5]. A similar phenotype of keratin 16 expression and a high percentage of cycling cells is found in exponentially growing keratinocyte monolayer cultures. In vivo, this hyperproliferative phenotype is usually associated with inflammatory changes in both the dermal and epidermal compartment. This was seen in diseased skin (e.g. psoriasis) as well as in experimental models [6].

Previously, we have described a new serine proteinase inhibitor (skin-derived antileukoproteinase, which was given the acronym of SKALP) that is also part of the regenerative/hyperproliferative phenotype [7-9]. SKALP is found in lesional psoriatic epidermis, but is virtually absent in normal epidermis. Initially, SKALP was shown to be a low molecular mass, cationic, heat-stable protein similar to elafin, an epidermal proteinase inhibitor that was subsequently described by others [10]. In addition, an elastase-specific inhibitor that is identical to SKALP has recently been reportedin bronchial secretions, although the exact cellular source in vivo remains obscure [11]. We cloned and sequenced the complementary deoxyribonucleic acid (cDNA) of SKALP [12], which proved that SKALP is identical to elafin [13]. We showed that SKALP, as expressed in cultured epidermal keratinocytes, is translated as a 12.3 kDa protein. Cleavage of the signal peptide yields a 9.9 kDa protein that is the major form found in cultured cells, as was confirmed by purification and N-terminal amino acid sequencing [12]. Recently we have been able to assign the SKALP gene to chromosome 20, region q12-q13 [14]. The gene has been given the approved name of *Protease Inhibitor, skin derived (SKALP)*, symbol: PI3, in the Genome Data Base

of the HUGO nomenclature committee.

SKALP inhibits at least three serine proteinases, namely human leukocyte elastase, porcine pancreatic elastase and human leukocyte proteinase 3 [8,15]. The exact biological function of SKALP is not known at present, although the substrate specificity for PMN-derived, elastolytic proteinases suggests that SKALP is involved in regulation of cutaneous inflammation, or protection against PMN-dependent tissue damage. However, it is very possible that other target enzymes (e.g. from the keratinocytes or dermal fibroblasts) exist. Since we have found that the 9.9 kDa molecule, purified from cultured cells, contains multiple functional transglutaminase substrate motifs [12], functions other than controlling proteinase activity cannot be excluded.

In previous studies SKALP was described biochemically [8,16] and characterized at the protein and DNA level [12]. We established the cellular source and localization in psoriatic epidermis [9], and recently we found that SKALP is differentially expressed in human epidermal tumors [17]. In the present study, we have investigated the expression of SKALP in dynamic models for keratinocyte growth and differentiation in vivo and in vitro. It is demonstrated that SKALP expression can be induced in normal human epidermis and in cultured keratinocytes.

MATERIALS AND METHODS

Chemicals

Methoxysuccinyl-alanyl-alanyl-prolyl-valyl-7-amino-4-methyl coumarin was obtained from Bachem, Bubendorf, Switzerland. All reagents for sodium dodecylsulphate/polyacrylamide gel electrophoresis SDS-PAGE, including prestained markers, were obtained from Bio-Rad laboratories, Richmond, CA, USA. Polyvinylidenedifluoride (PVDF) membrane was from Millipore, Etten-Leur, The Netherlands. Low molecular mass markers used for SDS-gels, goat-anti-rabbit IgG biotin conjugate, avidin-alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium, isoproterenol and iododeoxyuridine (IdUrd) were obtained from Sigma Chemicals, St. Louis, MO, USA. Swine-anti-rabbit

peroxydase, fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins (RAM-FITC) and mouse anti-IdUrd antibody were obtained from Dakopatts, Copenhagen, Denmark. Penicillin, streptomycin and trypsin were obtained from Gibco, Breda, The Netherlands. Epidermal growth factor (EGF) and hydrocortisone were from Collaborative Research Inc., Lexington, MA, USA. Dulbecco's minimal essential medium (DMEM) and F12 medium were obtained from Flow laboratories, Irvine, UK. Keratinocyte growth medium (KGM) and bovine pituitary extract were from Clonetics, San Diego, CA, USA. A synthetic peptide comprising amino acid 23 to 36 of SKALP was synthesized by Eurosequence, Groningen, The Netherlands; at the C-terminus a cysteine residue was added to enable coupling to a carrier protein. The sulfo-SMCC kit and Sulfolink gel were obtained from Pierce, IL, USA, and anti-involucrin (rabbit) from Biomedical Technologies Inc., Stoughton, MA, USA. RNA zol™ B was obtained from Cinna/Biotex Laboratories, Inc., Houston, TX, USA, and α^{32}PdCTP from Amersham, UK. The 'Living Skin Equivalent' (LSE) culture system was purchased from Organogenesis, Cambridge, MA, USA.

Recombinant SKALP/elafin was a kind gift from Dr Norman Russell, ICI Pharmaceuticals, UK. As a probe for ribosomal 28S RNA, a 2.1 kb *Eco*R1/*Bgl*II fragment was used (a kind gift from Dr Jan Bauman, TNO Rijswijk, The Netherlands). As a probe for glyceraldehyde phosphate dehydrogenase (GAPDH) a 1.1 kb *Pst*I fragment was used (a kind gift from Dr Wiljan Hendriks, Dept of Cell Biology and Histology, University of Nijmegen, The Netherlands).

Skin biopsies and tape stripping

Biopsies of normal, psoriatic and tape-stripped skin were taken under local anaesthesia with a keratotome. The skin samples were either fixed in buffered 4% formalin or stored at -20°C until use for extraction of SKALP.

Tape stripping of human skin is the repeated application and removal of adhesive tape, which produces a standardized injury of the epidermis by removal of the stratum corneum [18]. Keratotome biopsies of the

stripped test sites and of normal skin were taken at several intervals and processed for SKALP measurement, Western blots, RNA extraction or histology. For functional assays, the biopsies were homogenized in a glass-glass grinder in distilled water, and centrifuged at 8,000 g for 15 minutes. After measurement of SKALP activity (as described below), the supernatant was concentrated by vacuum evaporation, and subjected to SDS-PAGE and immunoblotting.

Approval of the local medical ethical committee was obtained for all experiments.

Functional measurement of SKALP activity.

Inhibition of leukocyte elastase activity, using the fluorogenic substrate methoxysuccinyl-alanyl-alanyl-prolyl-valyl-7-amino-4-methyl coumarin [19], was used to assay SKALP. In this assay SKALP activity is calculated as the percentage inhibition of 1 ng elastase; the amount required to reduce the elastase activity in the assay by 50% is defined as one unit of inhibitory activity.

Purification and N-terminal sequencing of SKALP

SKALP was purified from psoriatic scales using chromatofocusing, reversed-phase chromatography and gel permeation chromatography, according to previously described methods [8,12]. N-terminal gas-phase sequencing of this material was performed by Eurosequence, Groningen, the Netherlands [8].

Antisera

An antiserum against recombinant SKALP/elafin was obtained as described previously [9]. An antiserum against a synthetic peptide comprising amino acids 23 to 36 of SKALP was prepared by coupling the peptide to chicken ovalbumin via a C-terminal cystein residue, using the sulfo-SMCC procedure, according to the manufacturer's instructions. This conjugate was used for immunization of a rabbit, according to previously described methods [8]. The antiserum was affinity-purified using the synthetic peptide coupled to Sulfolink coupling gel, according

to the manufacturer's instructions. Preimmune serum was drawn as a control. The two antisera against recombinant SKALP/elafin and synthetic peptide gave identical staining patterns on histological sections. A monoclonal antibody against involucrin was obtained as previously described [20].

Immunohistology.

Air-exposed cell cultures and biopsies of normal, psoriatic and tape-stripped skin, were fixed in buffered 4% formalin for at least 24 hours and processed for embedding in paraffin. Sections (5 μm) were deparaffinized, rehydrated, preincubated with normal swine serum and incubated with anti-SKALP/elafin serum, raised either against recombinant SKALP/elafin or against the synthetic peptide. After incubation with peroxidase-conjugated swine-anti-rabbit Ig, the sections were developed with aminoethylcarbazole as the chromogenic substrate. Control staining was performed as indicated above, substituting anti-SKALP/elafin serum with preimmune serum of the same animal.

SDS-PAGE and Western blotting.

Supernatants of homogenates of cell cutures and skin biopsies were vacuum-evaporated to dryness, dissolved in non-reducing sample buffer and subjected to SDS-PAGE. Proteins were separated on a 16% polyacrylamide gel, using tricine as a trailing ion instead of tris-glycine. Gels were blotted on PVDF membranes, and proteins were detected immunologically using biotinylated goat anti-rabbit IgG, avidin-conjugated alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate, and nitro blue tetrazolium. A polyclonal rabbit antiserum raised against recombinant SKALP/elafin was used.

RNA isolation and Northern blot analysis.

Total RNA from cultured cells and samples of normal, psoriatic and tape-stripped skin were extracted with RNAzol B, as suggested by the supplier. For Northern blot analysis, 10 μg of total RNA was fractionated on a denaturing 1% agarose gel containing formaldehyde,

following standard procedures [21] and blotted by capillary transfer on a nylon membrane. After transfer, RNA was fixed to the membrane by ultraviolet irradiation (312 nm, 0.2 J/cm^2). Hybridization was performed in phosphate buffer as previously described, using a 0.42 kbp *Pvu*II/*Eco*RI fragment of the SKALP cDNA clone pGESKA as a probe [12]. Control hybridizations for equal loading were performed using a human 28S ribosomal RNA probe and GAPDH probe. All probes were labelled with ^{32}P by random priming, following standard procedures. Autoradiography was done for on X-Omat S film (Kodak, France) at -80°C with an intensifying screen.

Keratinocyte culture in the Rheinwald-Green system.

First passage human keratinocytes derived from the backs of healthy, adult volunteers were seeded on gamma-irradiated Swiss mouse 3T3 fibroblasts [22]. Growth medium was DMEM/F12 3:1 supplemented with 0.4 μg/ml hydrocortisone, 10^{-6}M isoproterenol, 100 U/ml penicillin plus 100 μg/ml streptomycin, 6% fetal calf serum and, starting at day three after seeding, 10 ng/ml EGF. The Ca^{2+}-concentration in the medium was 2 mM. Keratinocytes were grown at 37°C, 95% relative humidity and 7.5% CO_2. Cells were harvested by trypsinization, counted and used for SKALP measurement, Northern blot analysis or flow cytometry. Labelling with IdUrd was performed for 30 hours before harvesting the cells. Either exponentially growing cells, confluent cells or cells in suspension (see below) were used.

Keratinocyte culture in suspension.

Confluent keratinocyte cultures were trypsinized and resuspended in DMEM/F12 medium (without EGF), as described above. The cell suspension was kept in a continuously rotating device for 48 hours, as described before [23], and analysed as described above. This procedure induces terminal differentiation, as measured by involucrin expression, and gives results similar to the systems previously used by others [24,25].

Keratinocyte culture in KGM.

Human keratinocytes from healthy volunteers were cultured in serum-free KGM that was composed of keratinocyte basal medium (KBM) with 0.15 mM Calcium, supplemented with 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 0.4% bovine pituitary extract, 10 ng/ml EGF, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 100 U/ml penicillin and 100 μg/ml streptomycin. For induction of SKALP expression, cultures were switched at 30% confluency to KGM plus 5% fetal calf serum or KGM with additional Ca^{2+} (either 0.35 mM or 2 mM). Cells were harvested after 48 h by trypsinization and extracted for measurement of SKALP activity and Northern blot analysis.

Keratinocyte culture under air-exposed conditions.

Human keratinocytes derived from healthy individuals were first cultured using the Rheinwald-Green feeder technique, and subsequently seeded on de-epidermized dermis (DED) and grown under air-exposed conditions according to the Régnier-Pruniéras method [26]. Growth medium at air-exposed conditions was Dulbecco-Vogt and Ham's F12 3:1 supplemented with 0.4 μg/ml hydrocortisone, 10^{-6} M isoproterenol, 5% fetal calf serum, 10 ng/ml EGF [27].

The LSE model for air-exposed culture, consisting of human keratinocytes seeded on a fibroblast populated collagen gel, was cultured according to the manufacturer's instructions.

Immunocytochemical staining and flow cytometric analysis.

A standard indirect immunocytochemical labelling technique was used with both anti-involucrin and anti-IdUrd antibodies on ethanol-fixed cells. DNA staining was performed with propidium iodide. Flow cytometric analysis was carried out using previously described methods [23,28].

RESULTS

Expression of SKALP in vivo.

In previous studies we have shown that SKALP is expressed in the upper suprabasal layers of lesional psoriatic skin but is virtually absent in normal human skin. Here, removal of stratum corneum by tape stripping was used as a model for standardized epidermal injury in normal human skin. Fig. 1 shows a time curve of SKALP-activity in biopsies, measured as anti-elastase activity using a fluorimetric assay. At 48 hours a peak was found, and this time point was used to characterize SKALP on Western blots and for detection by immunohistochemistry.

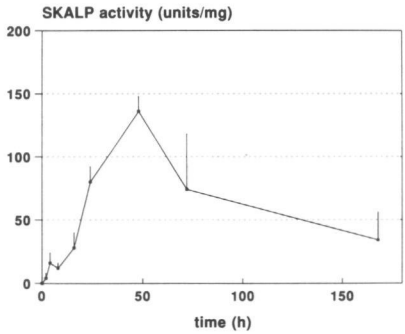

Figure 1. Time course of SKALP induction by tape stripping of the skin of healthy volunteers (n=6). SKALP activity is given in units per mg skin homogenate. Maximal SKALP activity is seen after 48 hours, standard error is represented by the vertical lines at each time of sampling.

In Fig. 2 it is demonstrated that, after tape stripping, one major low molecular mass form of SKALP is found, in contrast with findings in psoriatic epidermis and psoriatic scales, which contain multiple low molecular mass forms of SKALP.

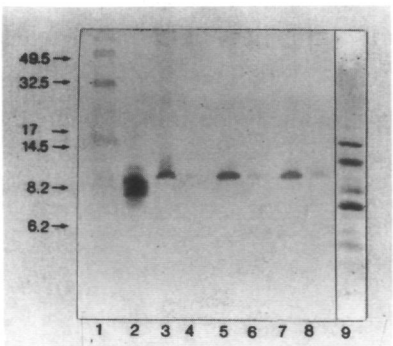

Figure 2. Western blot of extracts from psoriatic scales (lane 2), normal human skin (lanes 4, 6 and 8), and tape-stripped skin after 48 hours, taken from the same individuals (lanes 3, 5 and 7). Lanes 1 and 9: molecular mass markers. Staining with polyclonal rabbit antiserum against recombinant SKALP/elafin. Preimmune serum was negative with all samples (not shown).

In extracts of biopsies from normal skin only trace amounts of SKALP were detected, in accordance with previous findings [7]. N-terminal amino acid sequencing of SKALP purified from psoriatic scales gave 3 sequences (KGPVSTP, AQEPVKGP and VxAQxxVK) which correspond with SKALP fragments starting at amino acid 66, 61 and 59, respectively (numbering based on the 12.3 kDa translation product). The SKALP forms found in tape-stripped skin and in psoriatic epidermis could not be purified in sufficient quantities for N-terminal amino acid sequencing.

Immunohistological staining of skin after repeated tapestripping revealed 2-3 cell layers (mainly granular cells) with cytoplasmic staining positive for SKALP (Fig. 3a). This pattern is similar to that found in lesional psoriatic skin (Fig. 3b) with respect to the absence of SKALP staining in the basal layers; a difference with psoriatic skin is the absence of staining in cells of the stratum spinosum. Normal epidermis was totally negative (Fig. 3c).

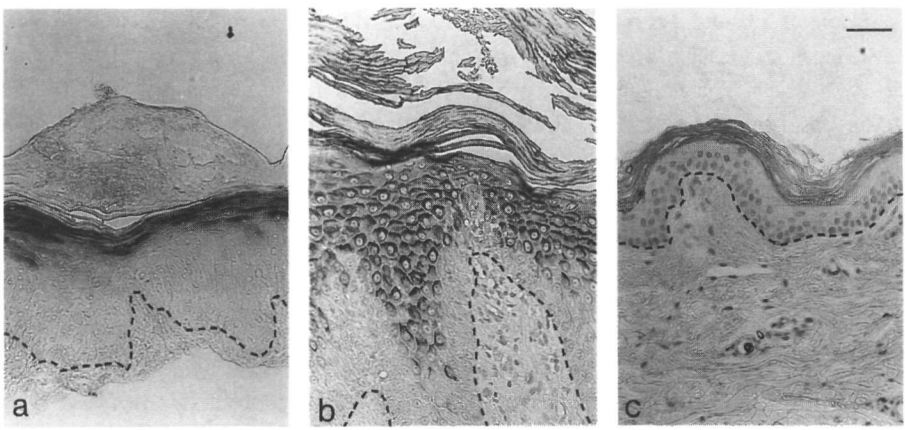

Figure 3. Immunohistology of tape-stripped skin (after 48 hours), psoriatic lesional skin, and normal epidermis. a. Tape-stripped skin. The staining is limited to the upper layers of the suprabasal compartment, which contains mainly granular cells. b. Psoriatic lesional skin. Note that several layers of suprabasal spinous cells of the psoriatic lesion are positive. Basal cells are negative. Non-lesional skin was also negative (not shown). c. Normal epidermis. No positive staining was seen in the epidermis. Staining with polyclonal rabbit antiserum against recombinant SKALP/elafin. Bar, 50 μm.

Fig. 4 shows the presence of a 0.8 kb messenger ribonucleic acid (mRNA) in biopsies taken from psoriatic skin and from normal skin 40 h after injury. Normal, uninjured skin is essentially negative for SKALP, even on an overloaded gel.

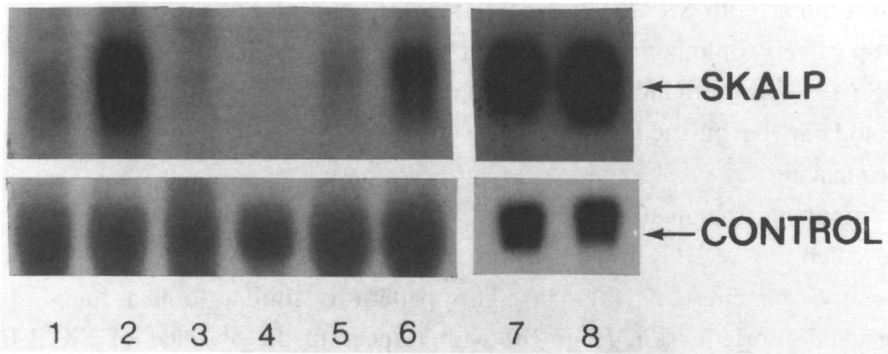

Figure 4. Northern blot of SKALP mRNA in skin biopsies and cultured keratinocytes (upper panel). 10 μg of total RNA was loaded. In biopsies from normal skin at 40 hours after injury (lane 1), and in skin from psoriatic patients (lane 2) a 0.8 kb message was found. Normal human epidermis was essentially negative (lane 3) even on overloaded (50 μg RNA) gels. Keratinocytes cultured in KGM were essentially negative (lane 4). Addition of 1% and 5% fetal calf serum (lanes 5 and 6) induced SKALP mRNA, in a dose-dependent fashion. Keratinocytes cultured on a 3T3 feeder layer give a strong signal (lane 7) which is slightly increased when cells are cultured in suspension (lane 8). The lower panel shows control hybridizations to check for equal RNA loading. In lane 1-6 a probe for 28S ribosomal RNA was used; in lanes 7 and 8 a GAPDH probe was used.

Expression of SKALP in submerged culture systems.

We have previously shown that keratinocytes cultured in the Rheinwald-Green system, using a feeder layer of irradiated 3T3 cells, produce SKALP in a high molecular mass form that contains both the transglutaminase substrate domain and the antiproteinase domain. To investigate modulation of SKALP expression, we studied exponentially growing cells, confluent cells and cells that were kept in suspension in order to induce differentiation. Fig. 5 shows the amounts of SKALP extracted from cells cultured under these conditions, as measured by functional anti-elastase activity. Under hyperproliferative conditions (high percentage of IdUrd-positive cells) a high amount of SKALP was found and a low degree of differentiation, as assessed by the percentage

involucrin-positive cells using flow cytometry. Keratinocytes in suspension leave the cell cycle (low percentage of IdUrd-positive cells) and show a rapid induction of involucrin, whereas the total amount of soluble SKALP decreases.

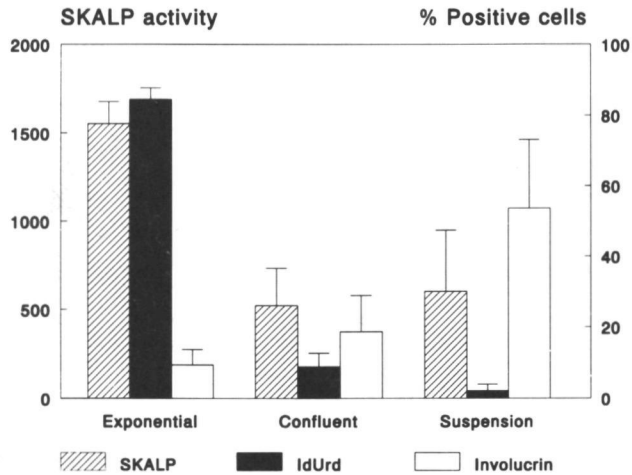

Figure 5. SKALP activity in cultured keratinocytes. Amounts of soluble SKALP extracted from exponentially growing cells, confluent cells or from cells that were kept in suspension are shown (left hand y axis). SKALP activity is expressed in units per 10^6 keratinocytes. Markers of proliferation (IdUrd) and differentiation (involucrin) were measured using flow cytometry, and the percentage of positive keratinocytes for these markers are shown (right hand y axis).

This is in contrast to Northern blot analysis, which showed a slight increase of SKALP mRNA in keratinocytes cultured in suspension (Fig. 4). The pattern of SKALP expression in this culture system is distinct from that found in vivo where basal, proliferating keratinocytes are negative for SKALP and the upper suprabasal, differentiated cells show SKALP expression.

Induction of SKALP expression in keratinocyte monolayers was studied in the KGM system, a serum-free, submerged culture system, using bovine pituitary extract as a source of growth factors. In KGM alone no significant SKALP expresssion was found either at the protein or at the mRNA level. However, addition of fetal calf serum could

induce SKALP expression in a dose-dependent fashion, as shown in Fig. 4, at the mRNA level. In a functional assay more than a 10-fold increase in SKALP activity was found 48 hours after the addition of 5% fetal calf serum (Fig. 6).

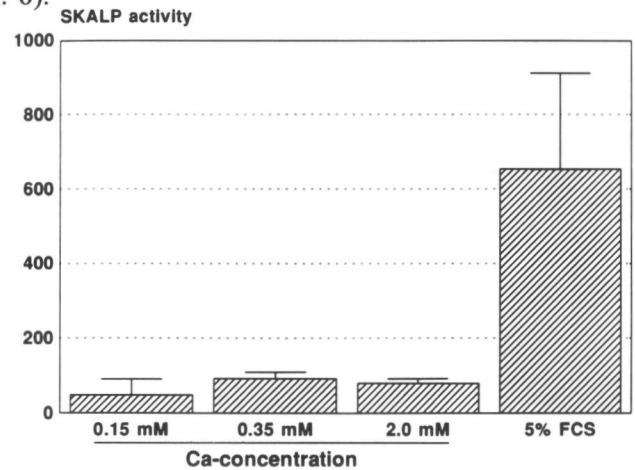

Figure 6. Induction of SKALP activity in submerged keratinocyte cultures using KGM. In unsupplemented KGM (0.15 mM Ca^{2+}) very little SKALP activity is found. Addition of 5% fetal calf serum induces SKALP, as measured after 48 hours, in a functional assay on cell extracts. Addition of $CaCl_2$ up to 2 mM did not induce a significant increase. SKALP activity is expressed as units per 10^6 keratinocytes.

As 5% serum raises the Ca^{2+}-concentration in KGM from 0.15 mM to approximately 0.35 mM, we tested the effect of additional Ca^{2+} in KGM. As shown in Fig. 6 no effect was noted up to 2 mM Ca^{2+}.

Expression of SKALP in air-exposed culture systems.

In submerged culture systems, as described above, keratinocyte phenotype and functional properties are distinct from the in vivo situation in normal skin. This has been demonstrated for several proteins including keratins, involucrin and transglutaminase. The expression pattern of SKALP in these systems does not correlate with the in vivo patterns found in psoriasis and tape-stripped skin, where SKALP is found in the differentiated compartment. We therefore investigated the expression pattern and processing of SKALP in the 'reconstructed skin' model, which approaches the in vivo situation. Cultures of human

epidermal keratinocytes seeded on DED were extracted for Western blottings and processed for immunohistology. Fig. 7 shows that high molecular weight forms of SKALP are present, with a predominance of the 9.9 kDa form also found in keratinocytes in the Rheinwald-Green system.

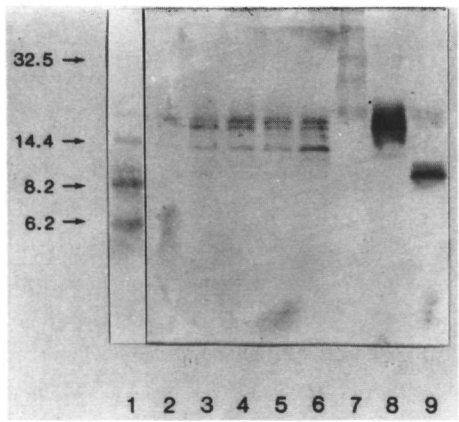

Figure 7. Western blot of cultures of human epidermal keratinocytes seeded on DED. Lanes 2-6, extracts of five different culture conditions: 10 days submerged; 3 days submerged and 7 days air-exposure; 3 days submerged and 5 days air-exposure; 3 days submerged and 2 days air-exposure; 3 days submerged, respectively. Lane 8, an extract from exponentially growing keratinocytes in the Rheinwald-Green system. Lane 9, recombinant SKALP/elafin. Lanes 1 and 7, prestained molecular mass markers. Cultured keratinocytes (lanes 2-6 and 8) contain high molecular mass forms of SKALP (including the 9.9 kDa processed form), whereas the 6 kDa form found in psoriatic scales (identical to recombinant SKALP/elafin) is absent. Note that the molecular masses given in the text are obtained from amino acid sequencing data. These values significantly deviate from the values obtained on SDS-PAGE, probably because of the cationic nature of SKALP.

The 6 kDa form, which is the major form in psoriatic skin, is absent in these cultures. No change in the pattern of SKALP processing is seen with time. Immunohistology shows that SKALP is barely detectable in submerged keratinocytes cultured on DED (Fig. 8a), but is moderately expressed in granular cells shortly after air exposure (Fig. 8b). In addition to the 'reconstructed skin' model, we examined SKALP expression in the commercially available LSE model, which uses a fibroblast-populated collagen gel as a matrix for keratinocytes.

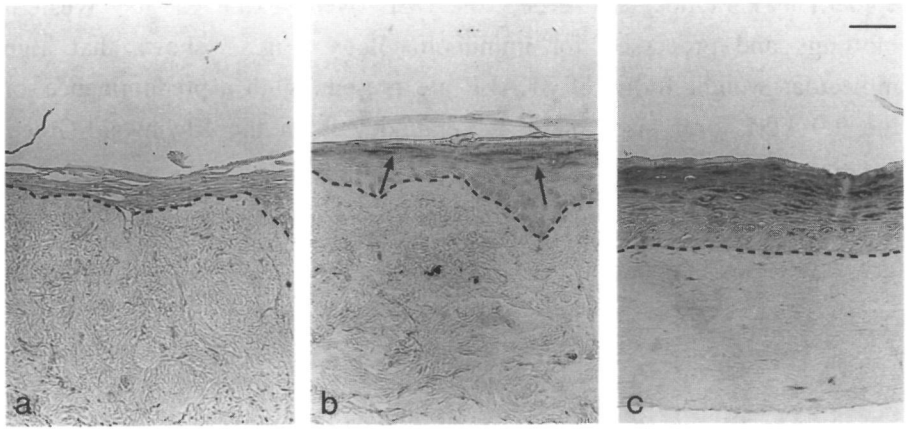

Figure 8. Immunohistology of cultures of human keratinocytes seeded on DED, and keratinocytes cultured on a fibroblast-populated collagen gel (LSE model). a. Culture of human keratinocytes seeded on DED, 10 days submerged. No significant SKALP expression was found. b. Culture of human keratinocytes seeded on DED, 3 days submerged followed by 3 days air-exposure. Scattered keratinocytes with a flattened, granular cell morphology were positive for SKALP (arrows). c. LSE model. SKALP is highly expressed in the upper suprabasal layers, similar to the in vivo expression in psoriatic and tape-stripped skin; see also Fig. 3 a. and b. Bar, 50 μm.

Fig. 8c shows that in this system SKALP is highly expressed in the upper suprabasal layers, similar to the in vivo expression of SKALP in psoriatic and tape-stripped skin.

DISCUSSION

We have examined the expression of SKALP by human epidermal keratinocytes using in vitro and in vivo models. It is shown that a transient wave of SKALP expression can be induced in normal human skin by epidermal injury. One major, low molecular mass form is found on Western blots. Positive immunohistological staining is found in cells of the granular layer. In vitro, the pattern and amount of SKALP expression depends on the culture system used. SKALP expression in the KGM sytem is inducible by fetal calf serum. In all in vitro systems tested, it is mainly the high molecular mass forms of SKALP that are

found.

Because of its specificity for the PMN-derived proteinases, elastase and proteinase-3, SKALP/elafin has been assigned a putative role in the control of epidermal inflammation [7,10]. Using cDNA cloning and N-terminal sequencing of SKALP purified from cultured keratinocytes, we have shown that the molecule contains a hydrofobic signal peptide and an additional functional domain with multiple transglutaminase substrate motifs [12]. Removal of the signal peptide results in a mature 9.9 kDa protein, which is the major form in cultured keratinocytes. However, in psoriatic skin, tape-stripped skin and in urine of psoriatic patients [16], low molecular mass forms predominate. Whether these forms result from interaction with PMN-derived proteinases, or from intracellular processing by keratinocyte-derived proteinases, remains to be investigated. The molecular masses, as indicated in the text, refer to calculated molecular masses obtained from amino acid and DNA sequence analysis, while apparent molecular masses on SDS-PAGE and Western blots are much higher (see Figs 2 and 7). This anomalous behaviour on SDS-gels is probably due to the highly cationic nature of the molecule.

At present it is not known which factors in vivo are responsible for SKALP induction. In psoriatic skin and in tape-stripped skin the expression pattern follows that of other differentiation-related markers such as involucrin and transglutaminase; a difference with these markers is the absence of SKALP in normal epidermis (at least epidermis of the trunk and the extremities). The expression pattern of SKALP more closely resembles that of cytokeratin 16, which is absent in normal epidermis but is induced under hyperproliferative conditions such as psoriasis, regeneration after injury, and in cell culture in the Rheinwald-Green system. SKALP expression in cell culture systems was found to be strongly dependent on the model used. High amounts of soluble SKALP could be found in exponentially growing keratinocytes in the Rheinwald-Green system, whereas exponentially growing cells in KGM produced very low quantities of SKALP. Addition of fetal calf serum to KGM was shown to induce SKALP expression, both at the protein level

and at the mRNA level. Obviously this finding does not rule out the contribution of extracellular matrix molecules from 3T3 fibroblasts, soluble 3T3 fibroblast factors or negatively regulating factors in KGM.

When cells cultured in the Rheinwald-Green system were kept in suspension (which induces terminal differentiation), the amount of soluble SKALP decreased, but no significant change was found at the mRNA level. Low SKALP activity in differentiated cells was not due to crosslinking of SKALP to the insoluble fraction, since the amounts of both soluble and particulate SKALP activity decreased in terminally differentiating keratinocytes (not shown). The discrepancy between Northern blot results and the SKALP content of the cells could be explained by secretion of SKALP in the medium of the suspension cultures. However, due to interference by serum proteins we have so far been unable to obtain accurate SKALP measurements in the culture media.

Using immunohistology, in the air-exposed culture systems, a moderate induction of expression was found in the 'reconstructed skin' model, shortly after air exposure. This induction was not found in Western blots of culture extracts, as shown in Fig. 7. However, the Western blots were primarily performed for qualitative analysis with respect to SKALP processing, and not for quantification of SKALP in the culture extracts. Histologically the expression pattern resembled that of tape-stripped skin, where positive SKALP staining was found mainly in flattened, granular cells. Using immunohistochemistry, a strong expression was observed in the LSE model. Possible explanation could be differences in the growth state of the cells, or stimulation of SKALP expression by fibroblast-derived factors (such as interleukin-6). Extrapolating the in vitro data to the in vivo situation, we speculate that induction of SKALP after tape stripping or inflammation in general, could be due to plasma derived factors. This notion is supported by a recent report showing that during inflammation, plasma protein levels are strongly increased in epidermis, which is otherwise devoid of plasma proteins [29].

The in vivo and in vitro models described here will be used to further identify the factors that induce SKALP expression. This will provide

relevant data on the cell biology of cutaneous inflammation as seen in pathological conditions (e.g. psoriasis) and the pathophysiology of wound healing.

ACKNOWLEDGEMENTS

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Chapter 4

Differential expression of skin-derived antileukoproteinase (SKALP) in normal human epithelia

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Submitted, 1994

ABSTRACT

Skin-derived antileukoproteinase (SKALP), also known as elafin, is a serine proteinase inhibitor that was first discovered in psoriatic epidermis. SKALP was absent in normal skin but later shown to be induced in other inflammatory skin disorders and in several epidermal tumors. SKALP inhibits leukocytic elastase and proteinase 3, both derived from polymorphonuclear neutrophils (PMN), which suggests that it plays a role in the regulation of the inflammatory response. Until now immunohistochemical localization studies have been restricted to normal and pathological human skin from various origins. Here we have studied the distribution of SKALP in a number of normal tissues.

Immunohistologically, SKALP appeared to be present in the following epithelial tissues: tongue, palate/lingual tonsil, gingiva, epiglottis, pharynx, esophagus, uterine cervix, vagina, and hair follicle. No SKALP could be observed in brain, cornea, larynx, bronchus, lung, duodenum, colon, liver, kidney, urethra, and normal epidermis of various locations. In general, stratified squamous epithelia are positive, in contrast to the pseudostratified epithelia, simple epithelia (glandular epithelia) and normal epidermis. The tissues that are positively stained are in direct contact with the environment subjected to a constant load of inflammatory stimuli. Low-grade PMN infiltration in some of these tissues is almost physiological, and was observed in many sections. The histological findings were largely confirmed by Northern blot analysis.

In view of the fact that SKALP inhibits PMN-derived proteinases, these results suggest that this molecule plays a role in the control of epithelial tissue homeostasis and integrity.

INTRODUCTION

In previous studies we have shown that the proteinase inhibitor skin-derived antileukoproteinase (SKALP), also termed elafin, is present in human epidermal keratinocytes under pathological conditions such as cutaneous inflammation or epidermal injury, but is virtually absent from

normal skin [1-3]. Cultured keratinocytes and some epidermal tumors also produce SKALP [4,5], and in bronchial secretions SKALP has been described by others as 'elastase-specific inhibitor' (ESI) [6]. SKALP has been characterized in biochemical and cell biological studies [4,7-9]. The cDNA and gene of SKALP have recently been cloned and sequenced, and the chromosomal localization has been assigned to chromosome region 20q12-q13 [9-11]. Although inhibition of the proteinases human leukocyte elastase and proteinase 3 points at an involvement in the regulation of cutaneous inflammation [1,12], the exact biological function is not known at present. Apart from a region with proteinase inhibiting properties, the SKALP molecule also contains a domain with transglutaminase substrate motifs [9]. Immunohistologically, a colocalization of SKALP expression and expression of transglutaminase is observed in hyperproliferative epidermis. Previous studies in epidermis, cultured keratinocytes, and human epidermal tumors showed that SKALP expression patterns appear to be similar to that of cytokeratin 16, which is known to be indicative for hyperproliferation [5,7,13,14]. However, since previous studies were restricted to inflamed epidermis and epidermal tumors, little is known about presence and localization in other human epithelial tissues.

The aim of this investigation was to study the expression of SKALP in normal human epithelia. The presence of SKALP in several but not all epithelial tissues is demonstrated and the differential expression is discussed.

MATERIALS AND METHODS

Chemicals

Goat-anti-rabbit IgG biotin conjugate, avidin-alkaline phosphatase, and the chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, and aminoethyl carbazole were obtained from Sigma Chemicals, St. Louis, MO, USA. Swine-anti-rabbit Ig conjugated with horseradish peroxidase (SWARPO) was obtained from Dakopatts, Glostrup, Denmark. A synthetic peptide comprising amino acid 23 to 36

of SKALP was synthesized by Eurosequence, Groningen, the Netherlands; at the C-terminus a cysteine residue was added for coupling to a carrier protein. The sulfo-SMCC kit and Sulfolink gel were obtained from Pierce, Rockford, IL, USA. RNA zol™ B was obtained from Cinna/Biotex Laboratories, Inc., Houston, TX, USA, and α^{32}PdCTP from Amersham, UK.

Recombinant SKALP was a kind gift from Dr. Norman Russell, ICI Pharmaceuticals, UK. As a probe for ribosomal 28S RNA, a 2.1 kb EcoR1/BglII fragment was used (a kind gift of Dr Jan Bauman, TNO Rijswijk, the Netherlands).

Histology

Archival biopsy material as well as autopsic material were selected. The following epithelial tissues were studied with respect to SKALP expression: brain; cornea; tongue; palate/lingual tonsil; gingiva; pharynx; larynx; epiglottis; vocal fold; lung; bronchus; esophagus; duodenum; colon; liver; kidney; urethra; uterine cervix; vagina; skin from various regions including head, ear, breast, axillar region, mamilla, inguinal region, pubic area, scrotum, finger, and foot sole; and hair follicle. The specimens were fixed in buffered 4% formalin for at least 24 h, dehydrated in ethanol, and embedded in paraffin.

Antisera

An antiserum against recombinant SKALP was obtained as described previously [3]. An antiserum against a synthetic peptide comprising amino acids 23 to 36 of SKALP was prepared by coupling the peptide via a C-terminal cystein residue to chicken ovalbumin, using the sulfo-SMCC procedure according to the manufacturer's instructions. This conjugate was used for immunization of a rabbit according to previously described protocols [7]. Control serum (pre-immune serum) was drawn before the immunization procedure. The antiserum against the synthetic peptide was affinity purified using the synthetic peptide coupled to Sulfolink coupling gel according to the manufacturer's instructions. The specificity of the antiserum was validated on Western blots as previously

described [7]. The two antisera against recombinant SKALP and against the synthetic peptide gave identical staining patterns in histological sections.

A rabbit polyclonal antiserum against secretory leukocyte proteinase inhibitor (SLPI) was a kind gift of Dr. Hans Kramps, C.D.I., Lelystad, the Netherlands.

Immunohistological staining for SKALP

Before staining, sections were deparaffinized, and pre-incubated with 20% normal swine serum in PBS for 15 min, followed by an incubation for 60 min with the polyclonal anti-SKALP serum (at a dilution of 1:500) in PBS with 1% bovine serum albumine (BSA). After rinsing in PBS, sections were incubated for 30 min with SWARPO (at a dilution of 1:50) in PBS with 1% BSA and 5% human AB serum. Subsequently, sections were rinsed in PBS and Na-acetate buffer, and developed using aminoethyl carbazole as chromogenic substrate. Counterstaining with hematoxylin was performed, and sections were dried and embedded in glycerol-gelatin solution.

Control staining was performed as indicated above, the polyclonal rabbit anti-SKALP serum being replaced with pre-immune serum of the same animal. Staining with anti-SLPI serum (at a dilution of 1:50) was carried out following the procedure described above.

RNA isolation and Northern blot analysis

Total RNA from different tissues obtained from autopsy was extracted with RNAzol B as suggested by the supplier. The following epithelial tissues were studied: tongue, pharynx, larynx, epiglottis, vocal fold, lung, duodenum, colon, liver, kidney, urethra, and inguinal skin. For Northern blot analysis, 10 μg total RNA was fractionated on a denaturing 1% agarose gel containing formaldehyde following standard procedures [15] and blotted by capillary transfer on nylon membrane. After transfer, RNA was fixed to the membrane by ultraviolet irradiation (312 nm, 0.2 J/cm^2). Hybridization was performed in phosphate buffer as previously described, using a 0.42 kbp *Pvu*II/*Eco*RI fragment of the SKALP cDNA

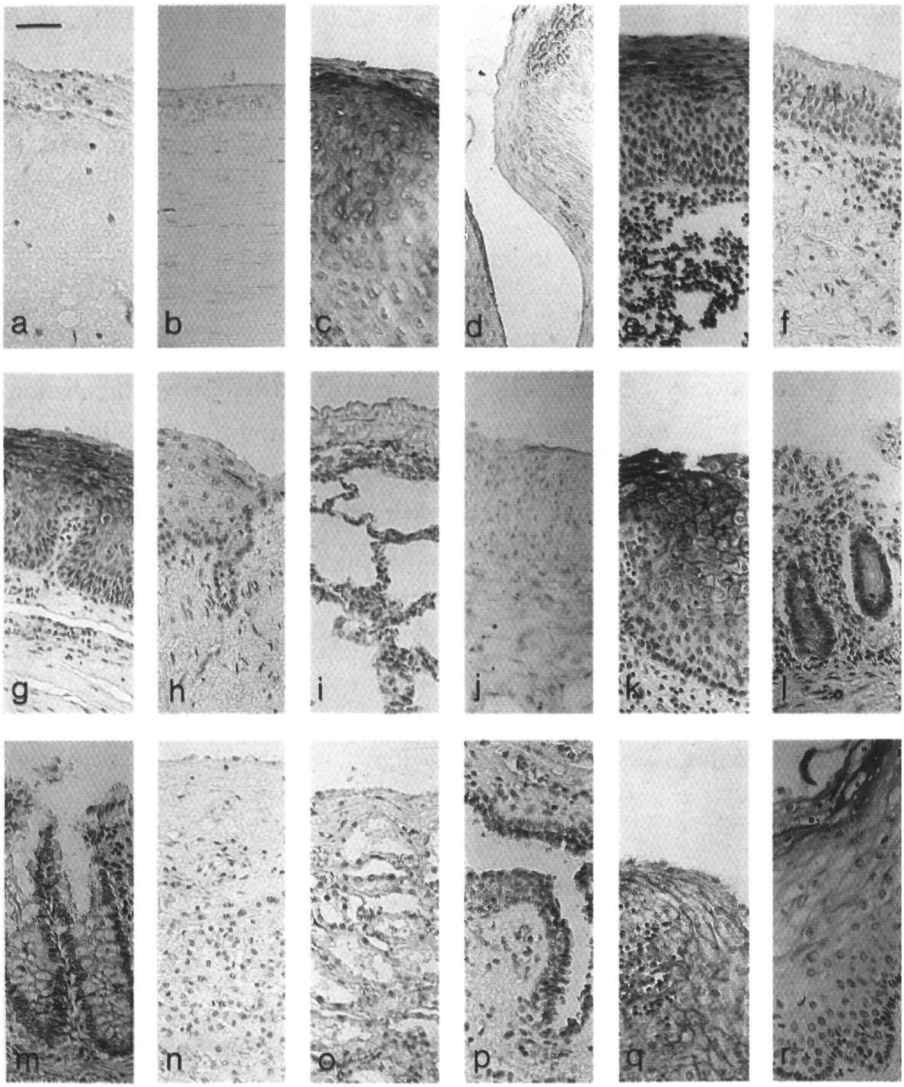

Figure 1. Immunohistology of various normal epithelial tissues. Staining with polyclonal rabbit anti-SKALP antiserum. a. meninges/gray matter (brain), b. stratified squamous epithelium (cornea), c. stratified squamous epithelium (dorsal part of the tongue), d. parakeratotic squamous epithelium (gingiva), e. stratified squamous epithelium (pharynx), f. pseudostratified ciliated epithelium (larynx), g. stratified squamous epithelium (epiglottis), h. stratified squamous epithelium (true vocal fold), i. alveoli (lung), j. pseudostratified epithelium (bronchus), k. stratified squamous epithelium (esophagus), l. crypt (duodenum), m. crypt (colon) n. mesothelium/parenchym (liver), o. cortex (kidney), p. urethelium (urethra), q. stratified squamous epithelium (uterine cervix), r. stratified squamous epithelium (vagina). Bar 50 μm.

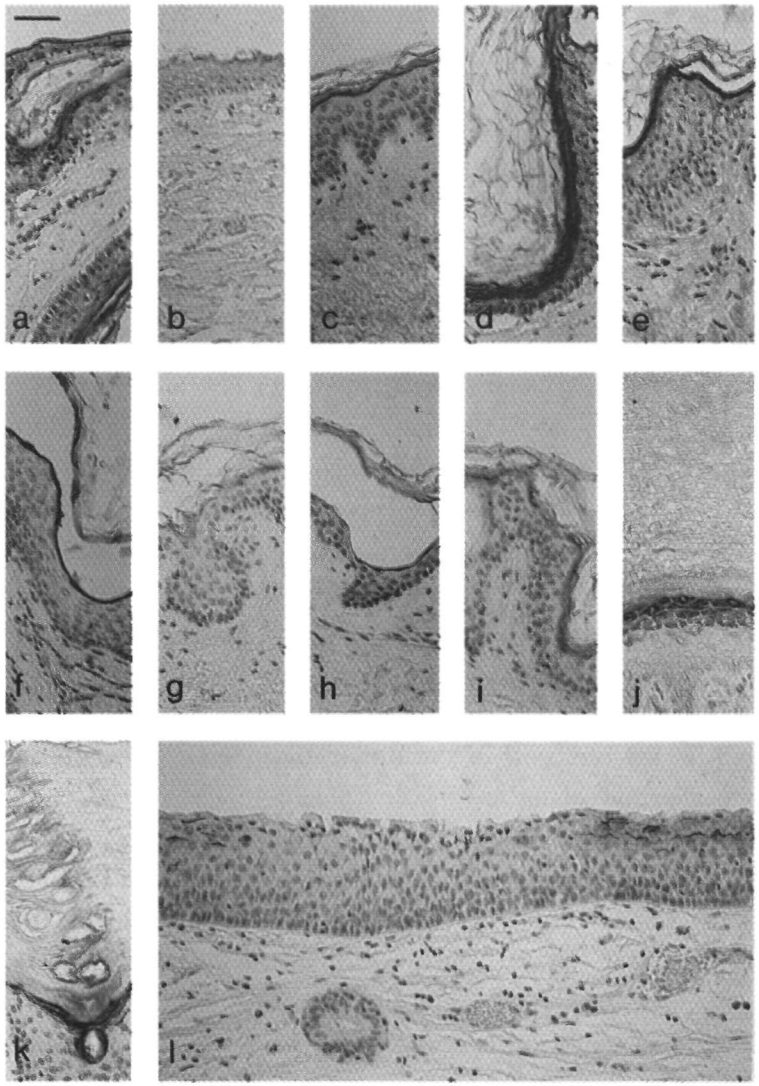

Figure 2. Immunohistology of various normal epithelial tissues. Staining with polyclonal rabbit anti-SKALP antiserum. a.-i. epidermis (skin). a. head, the infundibuli of the hair follicles are stained, the epidermis is negative, b. ear, c. breast, d. orifice of the hair follicle, axilla. Compare with e. where staining is absent, e. axilla without hair, f. mamilla, g. inguinal region, h. mons pubis, i. scrotum, j. finger tip, k. foot sole. Note that the excretory duct of an eccrine sweat gland is positive for SKALP staining, l. Transition of stratified squamous epithelium, positively stained, to pseudostratification with ciliated cells that are negative for SKALP expression (epiglottis). Bar 50 μm.

clone pGESKA as a probe [9]. Control hybridizations for equal loading were performed using a human 28S ribosomal RNA probe. All probes were labeled with ^{32}P by random priming following standard procedures. Autoradiography was performed on X-Omat S film (Kodak, France) at -80°C with an intensifying screen.

RESULTS

Immunohistology

We studied SKALP expression in the epithelial lining of 21 normal human tissues (see table I). Epithelial tissues that were positive with respect to SKALP expression were tongue, palate/lingual tonsil, gingiva, pharynx, epiglottis, vocal fold, esophagus, uterine cervix, vagina and the hair follicle. The expression varied considerably from one weakly stained cell layer to an increasing number of intensely stained layers (up to 10). Similar to the SKALP expression already described in pathological epidermis [3,5,16], staining was never seen in the basal cells. When multiple layers were stained, the density of the cytoplasmatic staining increased in the direction of the differentiated cells, often in a distinct apical localization in the cytoplasm (polarized appearance), whereas in other cases staining appeared to be localized close to the cell membrane.

The tongue dorsum was strongly positive: up to ten cell layers were intensively stained (Fig. 1.c). By contrast, the ventral part of the tongue was negative (not shown). The furrows surrounding the vallate papillae of the tongue stained intensively, but the taste buds were negative (Fig. 3.a). In palate/lingual tonsil a distinct SKALP expression was observed in destroyed epithelial lining, whereas staining was absent in the intact cover of the tonsil (Fig. 4). Keratinizing gingiva was moderately positive for three or four of the most differentiated cell layers (Fig. 1.d). The pharyngeal part showed a more or less patchy positive pattern, with SKALP expression in the epithelium overlying foci with inflammatory cells. Many layers with a varying staining intensity were observed in the positive areas (Fig. 1.e). The lining of the epiglottis was positively stained in multiple layers (Fig. 1.g). In between, areas of pseudostratified

columnar cells, with ciliated cells common to respiratory epithelia, were negative with respect to expression of SKALP (Fig. 2.l). True vocal fold was slightly positive (Fig. 1.h). Esophageal lining showed many positive cell layers, especially the superficial layers, intensively stained in a polarized pattern as already described (Fig. 1.k). The uterine cervix and vagina were positive for multiple cell layers, with varying intensity. The staining appeared to be more cell membrane associated than cytoplasmatic (Fig 1.q and r). The infundibular part of the hair follicles demonstrated a distinct SKALP expression (Figs. 2.a and 3.b).

Epithelial and non-epithelial tissues negative for SKALP expression included brain, cornea, larynx, lung, bronchus, duodenum, colon, liver, kidney, urethra, and skin from various body regions (see Material and Methods section) (Figs. 1 and 2).

SKALP shows considerable functional similarity and sequence homology to SLPI, a proteinase inhibitor in glandular tissues [6,7,17]. In order to ascertain whether a topographical overlap exists between SKALP and SLPI, we performed immunohistochemical staining for SLPI in a limited number of tissues. In psoriasis both anti-SKALP and anti-SLPI sera resulted in a positive signal, similar in pattern but different in intensity (Fig. 5.a and 5.b). As demonstrated in Fig 6, staining patterns were completely different for the two antisera in the tongue dorsum. The antiserum against SKALP gave a clear signal in the upper cell layers of the epithelial lining of the tongue (Fig. 6.a), whereas staining using the anti-SLPI serum was negative (Fig. 6.b). The minor salivary glands of the tongue, however, were positively stained with the antiserum against SLPI (Fig. 6.d), but were negatively stained with the antiserum against SKALP (Fig. 6.c).

These findings indicate that the polyclonal antisera against SKALP and SLPI are not mutually cross reactive. This was further checked on Western blots using recombinant SKALP and SLPI (data not shown). In addition to the antiserum raised against recombinant SKALP we used an antiserum against a synthetic peptide (12 N-terminal amino acids) from a region of SKALP that has no sequence homology to SLPI. The SKALP distribution patterns in immunohistology were identical for both antisera.

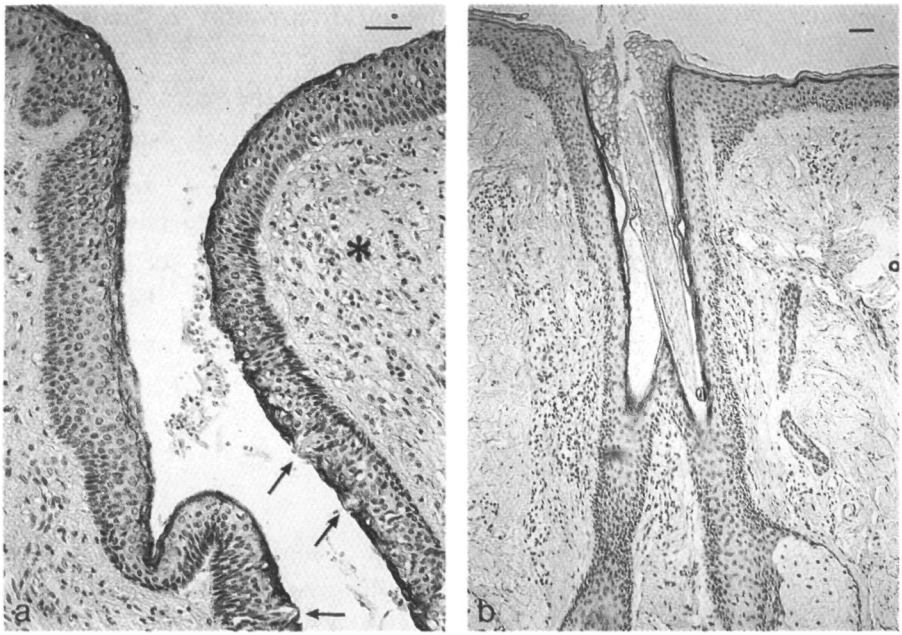

Figure 3. Immunohistology of a. part of furrow, surrounding lateral surface of vallate papilla (*), with negative taste buds (arrows), b. infundibulum of hair follicle. Staining with polyclonal rabbit anti-SKALP antiserum. Bar 50 μm.

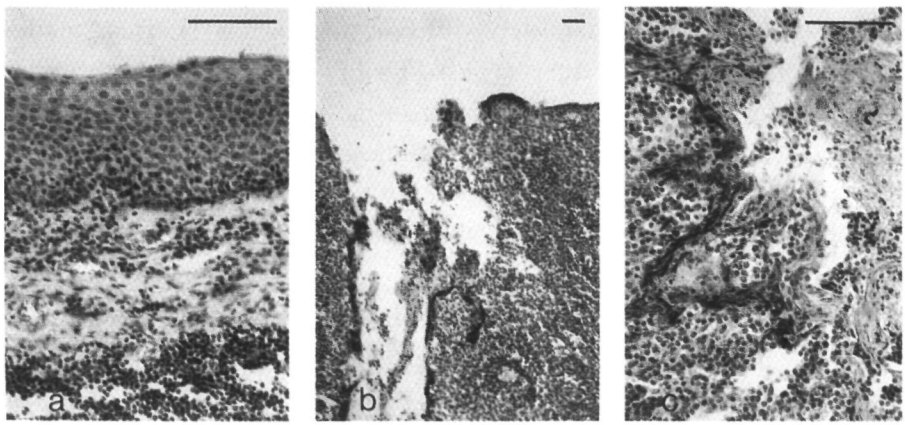

Figure 4. Immunohistology of the epithelial lining of the lingual tonsil. Staining with polyclonal rabbit anti-SKALP antiserum. a. Intact epithelial lining, negative. b. and c. The epithelial lining of the tonsillar crypt is positive. Lympho-diapedese causes desolution of the epithelial integrity, probably inducing SKALP expression. Bar 50 μm.

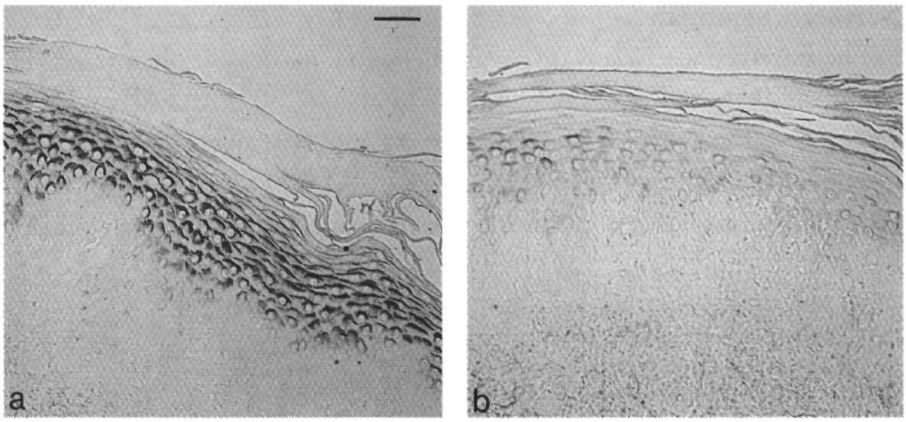

Figure 5. Immunohistology of psoriatic skin. a. Staining with a polyclonal rabbit anti-SKALP antiserum raised against a synthetic peptide comprising amino acids 23 to 36 of the SKALP molecule. b. Staining with rabbit polyclonal anti-SLPI antiserum. The staining pattern using the different antisera is similar, but the intensity is different. Bar 50 μm.

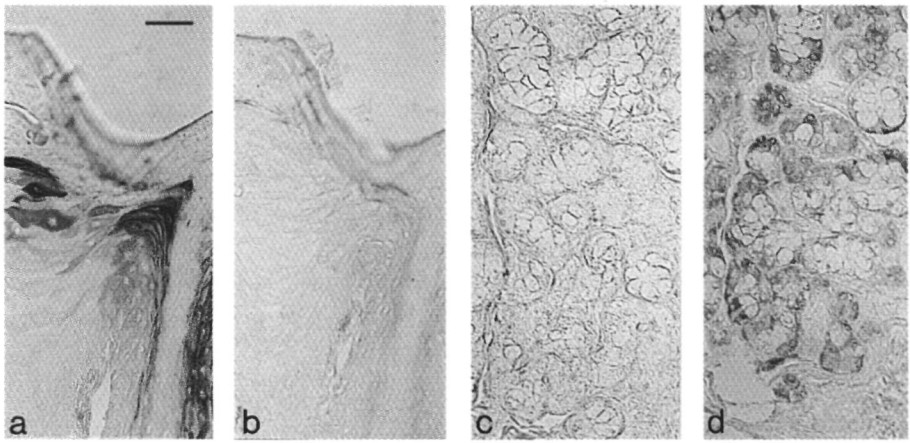

Figure 6. Immunohistology of the epithelial lining of normal human tongue. a and c. Staining with a polyclonal rabbit anti-SKALP antiserum. b and d. Staining with rabbit polyclonal anti-SLPI antiserum. Epithelial cover of the tongue (a and b) is positive for SKALP but negative for SLPI, whereas salivary glands (c and d) are positive for SLPI and virtually negative for SKALP. Bar 50 μm.

Northern blot analysis

Fresh autopsy material was obtained, stored at -80 °C, and used for Northern blot analysis. A strong expression of SKALP could be demonstrated in tongue and pharynx, and moderate levels were present in epiglottis and vocal fold (Fig. 7). No SKALP mRNA was found in larynx, lung, duodenum, colon, liver, kidney, urethra and inguinal skin.

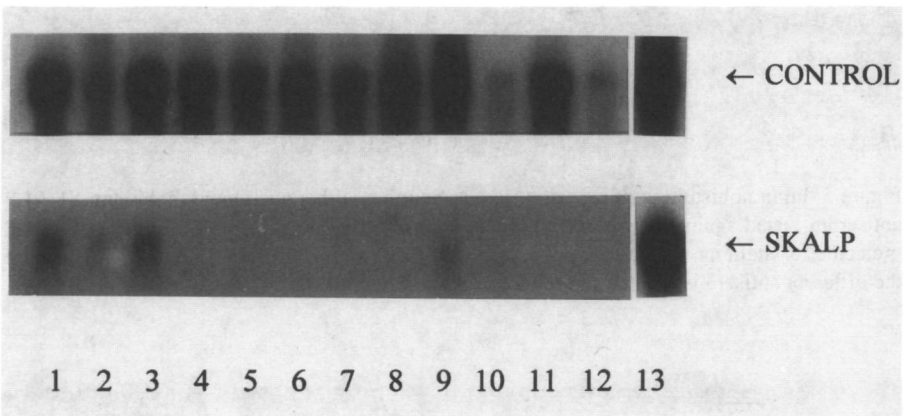

Figure 7. Northern blot of SKALP mRNA in autopsy material of various epithelial tissues. Lower panel. In tongue (lane 1), epiglottis (lane 2), pharynx (lane 3) and vocal fold (lane 9) and in lesional psoriatic skin which was used as the positive control (lane 13) a 0.8 kb message is found. Larynx, lung, kidney, urethra, skin of the inguinal region, and liver (lanes 4, 5, 6, 7, 8 and 11 respectively) are negative. Esophagus and bronchus mRNA (lane 10 and 12) could not be properly analyzed. In addition, colon and duodenum were analyzed and found to be negative (data not shown). In the upper panel a probe for 28S ribosomal RNA was used for control hybridizations to check for equal RNA loading.

DISCUSSION

The proteinase inhibitor SKALP is not present in normal skin, but is expressed by keratinocytes under hyperproliferative conditions, including psoriasis, epidermal injury, several skin tumors, and cell culture [1,4,9,13]. Under hyperproliferative or regenerative conditions, an alternative epidermal differentiation pathway is followed, and several proteins are expressed that are not normally present, such as the

cytokeratins 6, 16 and 17 [18,19]. In skin, the expression of these hyperproliferation-associated cytokeratins 6 and 16 is restricted to the suprabasal cells in lesions of psoriasis, actinic keratosis, the well-differentiated cells of squamous cell carcinoma, and epidermis adjacent to the tumor; basal epidermal cells do not show this K6/16 expression at the protein level [20]. In epidermis and epidermal tumors, SKALP is co-expressed with cytokeratin 16 [3,5,21], and is possibly subject to similar regulatory mechanisms. Some of the molecules that are expressed in epidermis during the regenerative differentiation program such as the cytokeratins 6 and 16 are also present in a number of normal tissues such as foot sole epidermis, hair follicle, and the lining epithelia of the tongue, esophagus and epiglottis [22]. Up to now, no data were available on the expression of SKALP in other tissues than skin.

Here we provide an immunohistological demonstration of the presence of SKALP in human tongue, tonsil (palate/lingual), gingiva, pharynx, epiglottis, esophagus, uterine cervix, vagina and the infundibular part of the hair follicle. When we compare these data with literature findings, it is clear that there is no absolute correlation between cytokeratin 16 and SKALP expression patterns, which suggests that they are differentially regulated at the tissue level.

It is shown that the distribution of SKALP is distinct from SLPI, which is a proteinase inhibitor with functional and structural similarities. Interestingly, SKALP and SLPI localization are mutually exclusive in tongue (Fig. 6). In psoriatic skin, however, SKALP and SLPI colocalize (Fig.5), which is in accordance with previous biochemical data on SKALP and SLPI present in psoriatic scales [1,2,23,24].

In contrast to the previous reports that dealt with SKALP present either in pathology or in cell culture, here we demonstrate that SKALP is expressed in 'normal' squamous epithelia. For a number of tissues the presence of SKALP is confirmed at the mRNA level. In general, a good correlation was found between SKALP expression or absence demonstrated by immunohistology and Northern blot analysis (see table I). The adjective 'normal' in this respect should be used with caution. Several 'normal' tissues are subjected to continuous mechanical stress or

inflammatory stimuli, which may influence the structure and organization of the lining epithelium. In normal epiglottis, squamous metaplasia can be observed that is partly functional or due to mechanical stress. Pathogens may be present in the pharynx of healthy individuals, and PMN infiltration is not considered to be pathological in normal pharynx and esophagus. Also in the infundibular part of the hair follicle the presence of bacteria that may induce an inflammatory response is common. SKALP expression was demonstrated in the tonsillar crypts where destruction of the epithelial lining by lymphocytes was observed (Fig. 4). Lymphocytes transmigrate the tonsillar epithelium into the crypts that also contain the desquamated cells of the stratified squamous epithelium. This passage is accompanied by desolution of the epithelium, which may trigger SKALP induction as part of the ongoing inflammatory response.

On the basis of the pattern of SKALP expression, one would surmise that SKALP expression is induced by inflammatory cells such as PMN. Interestingly, Sallenave *et al.* demonstrated induction of SKALP expression in tumor cell lines by the cytokines interleukin 1 (IL-1) and tumor necrosis factor (TNF), but also by the PMN-derived proteinases human leukocyte elastase and cathepsin G [25]. This finding is in line with that of Perlmutter *et al.*, who reported that neutrophil elastase regulates the synthesis of its inhibitor α1-PI in human monocytes and bronchoalveolar macrophages [26]. The cytokines IL-1 and TNF are considered to be initiating cytokines in inflammatory processes in cutaneous tissues [27]. As a result SKALP could be synthesized by the keratinocytes, and may hence be termed a local 'acute phase reactant' [1,25,28]. A single triggering signal for both PMN invasion and production of SKALP, which functionally counteracts PMN activity, is plausible since an unbalanced presence of elastase/proteinase-3 might cause unwanted tissue damage. In normal tissues where transient PMN presence is desired for reasons of host defense, this control mechanism might explain SKALP presence in epithelia such as esophagus, pharynx, tongue and vagina. The difference in SKALP expression in the various epithelia can be related to differences in mechanical forces and

inflammatory stimuli to which the tissues are subjected. Future studies are aimed at elucidating the mechanisms of SKALP induction *in vivo* and the functional significance of the molecule.

ACKNOWLEDGEMENTS

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Table I. SKALP expression on immunohistology and expression of SKALP mRNA on Northern blots of various normal epithelial tissues. +, positive for SKALP expression. −: negative for SKALP expression. n d.: not done. *: positive in most of the infundibuli of the the hair follicles if present in the sections.

Tissue	Immunohistology	Northern blot
Brain	−	n d
Cornea	−	n d
Tongue dorsal	+	+
ventral	−	n d
Palate/lingual tonsil	+	n d
Gingiva	+	n d
Pharynx	+	+
Larynx	−	n d
Epiglottis	+	+
Vocal fold	+	+
Lung	−	−
Bronchus	−	n d
Esophagus	+	n d
Duodenum	−	−
Colon	−	−
Liver	−	−
Kidney	−	−
Urethra	−	−
Uterine cervix	+	n d
Vagina	+	n d
Skin head	−*	n d
ear	−*	n d
breast	−*	n d
back	−*	−
axilla	−*	n d
mamilla	−	n d
inguinal regio	−*	−
mons pubis	−*	n d
scrotum	−*	n d
finger tip	−	n d
foot sole	−	n d
Hair follicle (infundibulum)	+	n d

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Chapter 5

Immunohistochemical localization of SKALP/elafin in psoriatic epidermis

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ABSTRACT

Recently we have reported the purification and biochemical characterization of a new, inducible elastase inhibitor [skin-derived antileukoproteinase (SKALP)], which could be extracted in high amounts from psoriatic skin but not from normal human skin. Here we demonstrate the immunohistochemical localization of SKALP in psoriatic epidermis. SKALP was found exclusively in the upper layers of the suprabasal compartment and stratum corneum of lesional psoriatic epidermis. Basal keratinocytes were always negative. No immunoreactive SKALP was found in normal epidermis and non-lesional psoriatic epidermis, in accordance with findings in functional assays. Western blots of skin extracts from psoriatic and normal skin confirmed the immunohistochemical findings and revealed two major bands with apparent molecular weights of 10.5 and 11.5 kDa.

We would hypothesize that SKALP could act as a modulator of epidermal inflammation by interfering with polymorphonuclear leukocyte trafficking, and that it could protect structural proteins against elastase-mediated damage.

INTRODUCTION

Neutral proteinases derived from polymorphonuclear leukocytes (PMNs) such as elastase, cathepsin G, and proteinase-3 have been implicated in a number of pathological processes [1], including lung emphysema [2], glomerulonephritis [3], arthritis [4,5] and inflammatory skin diseases [6]. Under non-pathological conditions, elastase and cathepsin G are controlled by specific high-affinity proteinase inhibitors such as alpha-1-proteinase inhibitor and alpha-1-antichymotrypsin in plasma, and secretory leukocyte proteinase inhibitor/antileukoproteinase (SLPI/ALP), which is produced locally in mucous tissues. Recently we have described a new, specific elastase-inhibitor in human epidermis that does not inhibit cathepsin G [7-9]. Using scales collected from patients with psoriasis, we purified and characterized this high-affinity inhibitor, which

was shown to be a cationic protein with an apparent molecular weight of 9 to 11 kDa. There is evidence that this molecule is derived from an 18-kDa protein, which is found in cultured keratinocytes and in variable amounts in psoriatic scales. Partial amino acid sequencing of SKALP [8] showed homology to SLPI/ALP [10]. SKALP is identical to elafin, a proteinase inhibitor which was recently described by others [11], on the basis of amino acid composition and amino acid sequence data.

Until now, only biochemical data were available on SKALP/elafin, and the exact source in human skin was not clear. In this article we studied the cellular localization of SKALP in psoriatic epidermis using immunohistochemistry; and it is shown that only a distinct subpopulation of the keratinocytes expresses this molecule.

METHODS

Chemicals

All reagents for sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Biorad laboratories, Richmond, CA. Aminoethylcarbazole, tricine, low-molecular-weight markers for SDS-PAGE, biotinylated swine-anti-rabbit immunoglobulin (Ig), avidin-peroxidase and avidin-conjugated alkaline phosphatase were from Sigma Chemicals, St Louis, MO. Swine-anti-rabbit Ig conjugated with horseradish peroxidase was obtained from Dakopatts, Glostrup, Denmark. Columns and materials for chromatographic purification of SKALP or anti-SKALP serum were from Pharmacia, Uppsala, Sweden. This included polybuffer exchanger 94, cyanogen activated sepharose 4B, protein A sepharose, a Superdex 75 FPLC-column, and a reverse-phase μRPC C2/C18 column.

Skin biopsies

Biopsies from psoriatic patients (n=9) and normal healthy volunteers (n=7) were taken with a keratotome or a razor blade, under local anaesthesia, and were either fixed in buffered formalin or stored at -20°C until used for extraction of SKALP. Permission from the local medical-

ethical committee had been obtained.

Extraction of SKALP from keratotome slices

Keratotome biopsies (5 to 10 mg) of psoriatic patients and healthy volunteers were homogenized in a glass-glass grinder in distilled water. After centrifugation (15 min at 12000 x *g*) the supernatant was boiled and again centrifuged. The supernatant was concentrated by vacuum evaporation, and subjected to SDS-PAGE and immunoblotting as described below. In addition, skin extracts were used for functional measurement of SKALP activity (anti-elastase activity) as described before [7-9]. For measurement of SKALP in the margin zone of the lesion, the keratotome biopsies of 7 patients were cut in 2-mm slices, which were extracted separately and assayed for anti-elastase activity.

We checked whether the total anti-elastase activity of these crude extracts could be attributed to SKALP, to exclude the possible interference by other elastase inhibitors such as alpha-1-proteinase inhibitor and SLPI/ALP. All elastase-inhibiting activity could be absorbed by sepharose 4B-conjugated immunoglobulins purified from anti-SKALP serum by protein-A chromatography. In addition, the assay conditions exclude the possible interference by alpha-1-proteinase inhibitor, and the amount of SLPI/ALP we found in psoriatic skin is far lower than SKALP (less than 5%).

Antiserum

The antiserum was raised against SKALP purified from psoriatic scales using chromatofocusing, affinity chromatography on immobilized pancreatic elastase, and finally Superdex-75 gel permeation fast protein liquid chromatography as described previously [8]. SKALP-preparations obtained by this procedure or alternatively via reverse-phase chromatography on a SMART μRPC-column as a final step appear to be homogeneous on chromatograms. However on SDS-PAGE multiple bands between 11 and 9 kDa (apparent molecular weight) can be seen, which probably represent truncated and extended forms of SKALP/lafin that were recently reported by Wiedow *et al* [12]. We could confirm

these findings by N-terminal sequencing, which showed that our preparation contains at least three elafin forms of different length (data not shown). N-terminal sequencing of a fragment generated by cleavage with pancreatic elastase from PVDF-blots [8] revealed substantial homology of SKALP with SLPI/ALP. However, the anti-SKALP serum did not crossreact with recombinant SLPI/ALP as shown before [9].

The antiserum was prepared in a rabbit which was selected to be devoid of reactivity to normal human skin. This was a necessary precaution since five of six rabbits had low titers of reactivity against the cytoplasm of normal epidermal kertinocytes (presumably keratins) or against basal membranes. Purified SKALP (50 μg) was crosslinked with glutaraldehyde, dialysed against distilled water, and emulsified with Freund's complete adjuvant. Rabbits were immunized intracutaneously in the back, followed by two subcutaneous boosters at weeks 2 and 4. Pre-immune serum was drawn before the experiments and immune serum was collected after 6 weeks. Titers in enzyme-linked immunosorbent assay were 1/20,000. For histology, dilutions between 1/100 and 1/1,000 were used. The antiserum was negative with extracts of normal human skin and human plasma proteins, on Western blots.

SDS-PAGE and Western blotting

Proteins were separated on a 16 % polyacrylamide gel, using tricine as a trailing ion instead of tris-glycine [8]. Low-molecular-weight markers from Sigma were used: Myoglobin (16950 Da), myoglobin fragment I+II (14440 Da), myoglobin fragment I+III (10600), myoglobin fragment I (8160), and myoglobin II (6210 Da). Gels were blotted on PVDF membranes and protein staining was performed with amido black according to standard procedures. Immunologic detection of proteins was performed using the antiserum described above, followed by biotinylated swine-anti-rabbit Ig and avidin-conjugated alkaline phosphatase. Pre-immune serum was used as a control.

Histology

Shave biopsies of human skin were fixed in buffered 4% formalin for at

least 24 h and processed for routine histology. Paraffin sections were made (5 μm) that were used for immunostaining according to standard protocols. Sections were deparaffinized, rehydrated, pre-incubated with normal swine serum, and incubated with anti-SKALP serum at a dilution of 1:100. After incubation with peroxidase-conjugated swine-anti-rabbbit Ig, the sections were developed with aminoethylcarbazole as the chromogenic substrate. Pre-immune serum from the immunized rabbit was used as a control.

RESULTS

Immunohistochemical localization of SKALP

Immunohistochemical staining of all normal skin specimens (n=7) was negative (Fig 1).

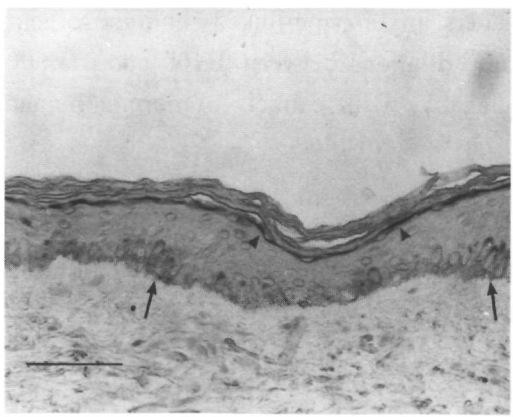

Figure 1. Immunoperoxidase staining of normal human skin with polyclonal anti-SKALP. Counterstained with hematoxylin and eosin. *Bar*, 50 μ. No positive staining was found. The darker cells are melanin contaning basal cells (*arrows*) and cells of the granular layer that pick up the counterstaining more intensely than the other cells (*arrowheads*).

The same was found for the non-lesional skin (n=7) of psoriatic patients (Fig 2). However, lesional psoriatic skin (n=9) revealed a subpopulation of the epidermal keratinocytes that was strongly positive for SKALP (Fig 3). The upper layers of the suprabasal compartment showed a strong cytoplasmic staining in all specimens studied, with minor interpatient variation. Staining of the upper parakeratotic layers and the stratum corneum varied from mild to strong. The wall of the cornified envelope was often found to be strongly positive, suggesting that SKALP is to

some extent crosslinked to the envelope proteins. No staining of basal cells or dendritic cells was observed in the epidermis. Also the dermal tissue was completely negative.

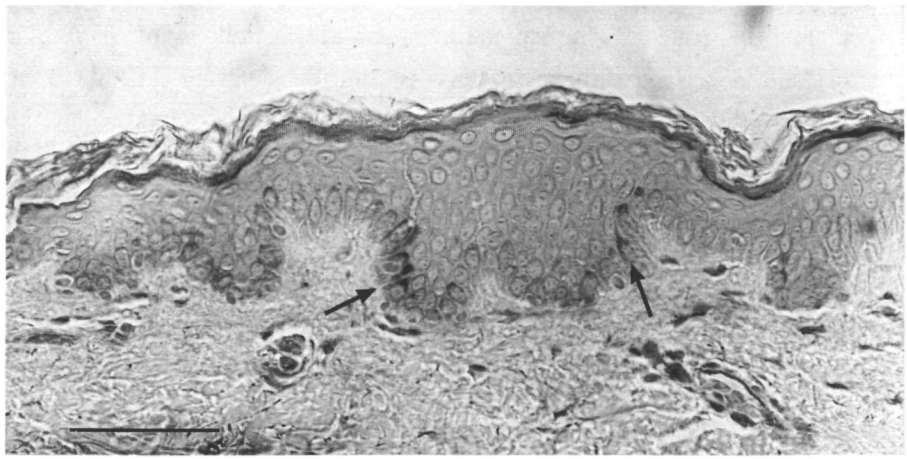

Figure 2. Uninvolved skin of a psoriatic patient. Staining as indicated in Fig 1. No positive staining was seen. The apparent staining of the basal cell layer is caused by melanin (*arrows*). *Bar*, 50 μ.

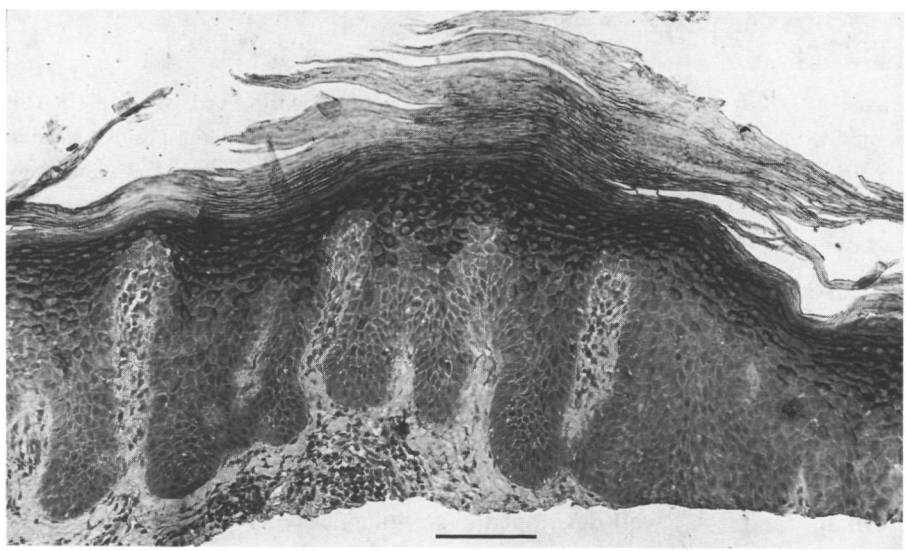

Figure 3. Lesional psoriatic skin, stained with polyclonal anti-SKALP serum. Note the strong, positive staining of the suprabasal keratinocytes. Also the cornified envelopes in the stratum corneum are stained. Hematoxylin and eosin counterstaining. *Bar*, 50 μ.

The control (pre-immune) serum was negative with all tissue samples studied (not shown). These immunohistochemical findings correlate with previous findings on the presence of SKALP in normal and psoriatic skin as determined with a functional biochemical assay [7].

The margin zone of the spreading psoriatic lesion is of particular interest since early pathogenetic events can be detected in this area. Previous studies [13] have shown that the pronounced changes in the lesional epidermis (such as parakeratosis, cytokeratin 16 expression, Ki-67 binding) show a clear transition to clinically uninvolved skin. It was found that SKALP expression declined when the morphology of the epidermis became normal (no acanthosis, no parakeratosis, and a well-developed granular layer). The transition, however, is not sharp. In Fig 4, a biopsy taken from the margin zone is shown.

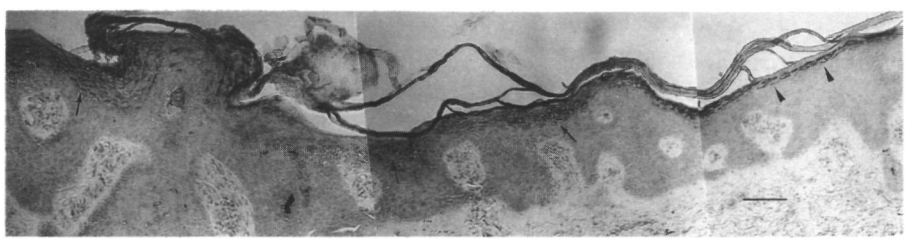

Figure 4. Biopsy from the margin zone of a psoriatic lesion, stained with anti-SKALP serum. On the left hand side a typical psoriatic morphology is seen with positive staining for SKALP in the upper spinous layers (*arrows*). On the right hand side the morphology resembles that of normal skin with little acanthosis and a well-developed graular layer (*arrowheads*). No positive staining for SKALP in the spinous layer is found. Hematoxylin and eosin counterstaining. *Bar*, 50 μ.

Positive staining for SKALP is seen in the suprabasal cells of the lesional part of the biopsy where acanthosis and scaling are visible and a granular layer is absent. At the other end of the biopsy no positive staining for SKALP is present; here the epidermal thickness is nearly normal and a well-developed granular layer is present. The immunohistochemical distribution correlated with previous findings in biochemical assays. SKALP activity in the lesion was 302 $\pm$ 182 units per mg, in the margin zone 52 $\pm$ 37 units per mg and just outside the

lesion in the clinically uninvolved skin 4 ± 4 units per mg tissue (mean of seven keratotome biopsies ± SD).

Western blots of skin extracts

The presence of SKALP in lesional psoriatic skin could also be demonstrated on Western blots. Extracts of keratotome biopsies were subjected to SDS-PAGE and blotted on PVDF membrane. Staining with anti-SKALP revealed two major bands of approximately 10.5 and 11.5 kDa, using the Sigma low-molecular-weight standards (Fig 5).

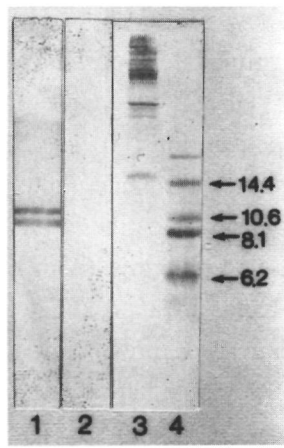

Figure 5. Western blot of proteins extracted from psoriatic lesional skin. *Lane 1*, staining with polyclonal anti-SKALP; *lane 2*, preimmune (control) serum; *lane 3*, Amido black staining of blotted proteins; *lane 4*, marker proteins. Molecular weights in kDa.

The staining pattern matches the pattern found for SKALP found in urine [9]. In SKALP preparations from psoriatic scales usually more heterogeneity in molecular weight is found. Normal human skin and non-lesional psoriatic skin were negative for SKALP (not shown). The preimmune serum was negative with all tissues studied.

DISCUSSION

In this article we demonstrate that SKALP/elafin, a new epidermal elastase inhibitor that was recently described by two groups at the biochemical level [7,11], is localized in the epidermal keratinocyte of psoriatic skin. In accordance with previous functional assays [7], no SKALP could be demonstrated in normal human epidermis. Surprisingly,

SKALP is only expressed in the suprabasal cells; basal cells are completely negative. The pattern of expression resembles the pattern found for differentiation-related proteins in psoriatic skin such as involucrin and transglutaminase [14]. A difference with these molecules is the fact that SKALP is lacking in normal skin, whereas involucrin and transglutaminase are expressed in the granular layer of normal skin but are prematurely expressed in the spinous layers of psoriatic skin.

Originally, SKALP/elafin was purified from psoriatic scales, and Wiedow *et al* (11) initially reported a molecular weight of 7017 for a 57-amino-acid molecule, as determined by N-terminal gas-phase sequencing. Recently Wiedow *et al* reported that at least five forms of elafin existed due to N-terminal deletions and extensions of the original 57-amino-acid molecule [12]. We have also consistently found heterogeneity in scale preparations; even SKALP preparations that appear pure on reverse-phase chromatography were found to contain mixtures of at least three SKALP peptides (not shown). As shown in this paper, in extracts of psoriatic epidermis (with only a minimal amount of scale present) two major bands of approximately 10.5 and 11.5 kDa are present. A similar finding was previously reported for urine from psoriatic patients [9]. In previous papers we reported the presence of SKALP in cultured human keratinocytes, with an apparent molecular weight of 18 kDa. We have recently completed the purification and N-terminal sequencing of this protein, which is indeed identical to SKALP/elafin but contains an additional 38 amino acids at the N-terminal side compared to the original 57-amino-acid molecule. These findings were confirmed at the cDNA level (Molhuizen *et al*, manuscript in preparation). Interestingly the elongated form of SKALP/elafin contains multiple motifs that are a consensus sequence for transglutaminase crosslinking. This might explain the immunohistochemical observation that SKALP is associated with the cornified envelope (see Fig 3).

Although there is now firm evidence for a role of the keratinocyte in the initiation of inflammation [15], little is know about the mechanisms that control inflammation. In this paper we demonstrate that epidermal

keratinocytes can produce a proteinase inhibitor that could act as an off-switch mechanism for the acute (PMN-dependent) phase of cutaneous inflammation. We have shown that SKALP is a molecule not present in normal skin. However, SKALP can be induced in normal skin following injury, and it is found in the lesional skin of psoriasis and other inflammatory dermatoses [7,16]. Speculatively, SKALP could function as in inhibitor of PMN migration and provide protection against elastase-mediated damage. SKALP could control the proteolysis of structural proteins in the epidermis (e.g., desmosomes) thereby preventing intraepidermal blister formation. It is very likely that SKALP also reaches the dermal compartment, because we have recently demonstrated SKALP in urine of psoriatic patients that is presumably derived from the epidermis [9]. Therefore, SKALP could also protect the dermal elastin fibers against proteolytic attack.

These findings suggest a novel and intriguing contribution of keratinocytes in the regulation of cutaneous inflammatory processes.

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Chapter 6

Demonstration of skin-derived antileukoproteinase (SKALP) in urine of psoriatic patients

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ABSTRACT

Recently we described a new elastase inhibitor (Skin-derived antileukoproteinase, SKALP) that is expressed in psoriatic epidermis and cultured keratinocytes, but is virtually absent in normal skin. In this study we investigated whether SKALP activity could be measured in urine of psoriatic patients and healthy controls. We found that urine of psoriatic patients contained considerable amounts of anti-elastase activity, whereas this activity in urine from normals was significantly lower. The properties of the urinary anti-elastase activity in psoriatic patients were indistinguishable from that of epidermal SKALP. It was found to be a cationic, heat-stable protein with an apparent molecular weight of 11 kDa on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and a K_i of approximately 2×10^{-11} M. In addition, in Western blotting partially purified inhibitor from urine was found to react with a polyclonal anti-SKALP serum. SKALP in urine was either present in a free form or in a latent form, most likely complexed with elastase. We speculate that SKALP in urine of psoriatic patients is derived from the epidermis, and that it might serve as a marker for disease activity.

INTRODUCTION

Polymorphonuclear leukocyte (PMN)-derived proteinases, including elastase, cathepsin G, proteinase III and collagenase, are known to cause tissue damage by attacking a variety of macromolecular substrates [1]. Proteinase inhibitors, either systemically present (e.g., α1-proteinase inhibitor, α2-macroglobulin) or locally synthesized (e.g., antileukoprotease (ALP) or secretory leukocyte proteinase inhibitor (SLPI)), are able to limit this proteinase action [2-4]. Disturbance of the local proteinase-antiproteinase balance is associated with several diseases, including pulmonary emphysema [5,6], arthritis [7,8], glomerulonephritis [9], cutaneous vasculitis [10] and several bullous dermatoses [11]. Recently we have described a new elastase-specific

proteinase inhibitor (SKALP) that is found in psoriatic epidermis and in cultured keratinocytes [12,13]. SKALP is very similar if not identical to Elafin, a skin-derived elastase inhibitor described by others [14,15], and to an elastase-specific inhibitor from sputum [16]. We have previously shown that anti-elastase activity can be found in scales from various dermatoses [17]. Normal skin, however, was found to contain very little, if any, anti-elastase activity [12]. Because anti-elastase is found to be inducable in normal skin following tape-stripping [12,18], it was concluded that it acts as a regulatory mechanism to control elastase-mediated epidermal tissue damage.

In this study we show that urine of psoriatic patients contains appreciable amounts of anti-elastase activity, either free or in a latent form. This anti-elastase activity was indistinguishable from SKALP, using biochemical and immunological criteria.

MATERIALS AND METHODS

Chemicals

Methoxysuccinyl-a lanyl-alanyl-prolyl-valyl-7-amino-4-methylcoumarin, was obtained from Bachem, Bubendorf, Switzerland. Cetyltrimethyl ammoniumbromide (CTAB) was obtained from ICI, UK. All reagents for sodium dodecylsulphate polyacrylamide gelelectrophoresis (SDS-PAGE) including prestained markers, were obtained from Biorad laboratories, Richmond, CA. Polybuffer exchanger 94 (PBE 94) was obtained from Pharmacia fine Chemicals, Uppsala, Sweden, and polyvinylidenedifluoride (PVDF) membrane from Millipore, Etten-Leur, The Netherlands. Swine-anti-rabbit peroxidase was obtained from Dakopatts, Copenhagen, Denmark. Low molecular weight markers used for SDS-gels and diaminobenzidine were obtained from Sigma Chemicals, St. Louis, MO.

Sample collection and preparation

Urine was collected from patients with severe psoriasis and volunteers without skin diseases, and stored at -20° C. The patient group consisted

of 16 men and 17 women, mean age 45 ± 20 years. The control group consisted of 7 men and 3 women, mean age 38 ± 12 years.
Urine samples were assayed for anti-elastase activity as described below. Elastase activity was measured both in untreated urine and urine that had been boiled for 2 min.

For preparative purposes, 40 ml urine was concentrated by vacuum evaporation, dialyzed against distilled water, and used for chromatographic purification.

Assay of elastase and anti-elastase activity

Elastase was measured according to previously described methods using the fluorogenic substrate methoxysuccinyl-alanyl-alanyl-prolyl-valyl-7-amino-4-methylcoumarin [9]. Inhibition of elastase activity was measured as the percentage inhibition of a standard amount of elastase. A sensitive microassay was used to enable measurement of small amounts of inhibitory activity. Briefly, 10 µl of 4 nM leukocyte elastase was mixed with urine sample (10 µl) and incubated at 37°C for 15 minutes to allow complexing of enzyme and the inhibiting component in the urine. Thereafter, 20 µl of 250 µM substrate solution was added and the reaction was allowed to proceed for 15 min at 37° C. All components were dissolved in 1 M NaCl, 0.4% CTAB, 0.1 M Tris pH 8.5. The incubation was terminated by the addition of 1 ml buffer (100 mM Na_2CO_3, pH 10.5) and fluorescence was measured at 375 nm (excitation) and 440 nm (emission) in a Perkin-Elmer LS-5 fluorimeter. Inhibition of elastase activity in this assay was calculated as the percentage inhibition of the activity of 40 fmol elastase (quantity in the assay). The unit of inhibitory activity was defined as the amount required to reduce the elastase activity in the assay by 50%. Elastase activity was linear during the assay (final substrate concentration 125 µM, of which less than 10% is consumed during the 15 min incubation). For all urine samples a dilution curve was made starting from a 1:2 dilution in assaybuffer (see also Fig 1). The amount of anti-elastase activity was expressed in units. When maximal inhibition was less than 50%, anti-elastase activity was considered to be undetectable. Thus the actual detection limit is 2 units

per 10 µl of urine.

Chromatographic procedures

A PBE 94 chromatofocusing column (6 x 1.4 cm) was equilibrated with 0.025 M ethanolamine buffer pH 9.4 (starting buffer), and 1-ml sample of concentrated, dialysed urine was applied in starting buffer. After sample application the column was washed with 35 ml of starting buffer. As previously described, this column can be used as a convenient and efficient purification step without the need to generate a pH gradient over the column [13]. SKALP is extremely cationic and elutes in the void volume of the column, whereas most other proteins are bound. The column was regenerated by 1 M NaCl.

The eluent was monitored at 280 nm, 2.5-ml fractions were collected and anti-elastase activity in the eluted fractions was measured as described above, expressed in units inhibitory activity per fraction. The fractions with elastase inhibiting activity were further concentrated by vacuum evaporation and used for kinetic studies, gel permeation chromatography, and SDS-gel electrophoresis.

Determination of dissociation constant K_i

Kinetic parameters were determined according to the criteria posed by Bieth [19], using active-site titrated leukocyte elastase, which was also used for determination of urinary SKALP concentrations. The dissociation constant K_i was determined by an Easson-Stedman plot based on the equation $I/(1-a) = K_i/a + E°$, where a is the fractional activity (ratio of enzyme activity in the presence and absence of inhibitor), $E°$ is the initial enzyme concentration, and I is the inhibitor concentration. The plot of $I/(1-a)$ vs. $1/a$ yields a straight line whose slope is K_i.

Determinations were made in 0.1 M Tris, 1M NaCl, and 0.4% cetyltrimethylammonium bromide, pH 8.5, as described before. The use of detergent and high salt is a *conditio sine qua non*, in order to prevent nonspecific absorption of inhibitor or enzyme, because extremely low concentrations of purified protein are used.

SDS-PAGE and Western blotting

Fractions with elastase inhibiting activity obtained after chromatofocusing were pooled, concentrated by vacuum evaporation, and subjected to SDS-PAGE (non-reducing conditions). Proteins were separated on a 16% polyacrylamide gel, using tricine as a trailing ion instead of tris-glycine [20]. This system has an improved resolution for low molecular weight molecules. Gels were blotted on PVDF membranes or sliced in order to elute SKALP from the gel. To this end, 2-mm slices of SDS-gel were extracted for 30 min in buffer and assayed as described above. Protein staining on PVDF membranes was performed with amido black according to standard procedures. Using a rabbit antiserum raised against highly purified SKALP from psoriatic scales, immunological detection of proteins was performed as described before [13]. A polyclonal antiserum against ALP/SLPI was a kind gift of Dr. J.A. Kramps, Department of Pulmonology, Academic Hospital Leiden, The Netherlands.

RESULTS

Measurement of anti-elastase activity in urine

In preliminary experiments we noticed that in urine from psoriatic patients variable amounts of anti-elastase activity could be demonstrated, and even small amounts of free elastase activity were occasionally found. Due to the complex situation of both elastase and inhibitor being present, a direct quantification of the amount of biologically active inhibitor was not possible. Therefore we developed a protocol for measurement of the total (latent plus free) anti-elastase activity in urine, by making use of the differential heat stability of elastase and inhibitor. By boiling the urine for a short period, elastase is denatured and heat-stable inhibitors are released from the enzyme-inhibitor complex. Figure 1 shows an example of urine from a psoriatic patient that contained a small amount of free elastase activity. When the urine was boiled and samples were drawn at various time intervals, it was found that the elastase activity disappeared (within 1 min) and that anti-elastase activity

was released. The inhibition curves of urine samples taken at t = 0,1,2,4 and 8 min as depicted in Fig 1, show that 2 min boiling gives an optimal recovery of heat-stable anti-elastase activity.

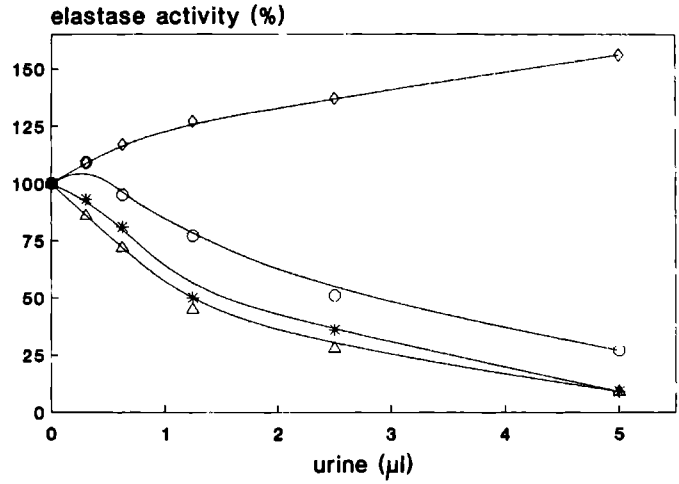

Figure 1. Dilution curves showing the effect of boiling on elastase and anti-elastase activity in urine from a psoriatic patient. When untreated urine was added to a standard amount of human leukocyte elastase (100% value), additional elastase activity was measured (*diamonds*). Boiling the urine samples abolished all elastase activity and released inhibitory activity in a time-dependent manner. Boiling was done for 1, 2 and 8 min, represented by *stars*, *triangles* and *circles*, respectively. Prolonged boiling resulted in a gradual decrease of inhibitory activity.

Using this protocol, heat-stable anti-elastase activity in the urine of 33 psoriatic patients and 10 healthy controls was measured as described in the *Materials and Methods* section. Table 1 shows the values of all patients and controls. Most of the psoriatic patients showed urinary anti-elastase activity. In urine of healthy controls anti-elastase activity was undetectable in all but one cases.

Characterization of urinary anti-elastase activity

In order to investigate its possible identity to SKALP, the urinary anti-elastase activity was further characterized by its behavior on a PBE 94 chromatofocusing column.

Figure 2 shows that most of the activity elutes in the void volume, indicating that it is a cationic molecule, very much like SKALP [13].

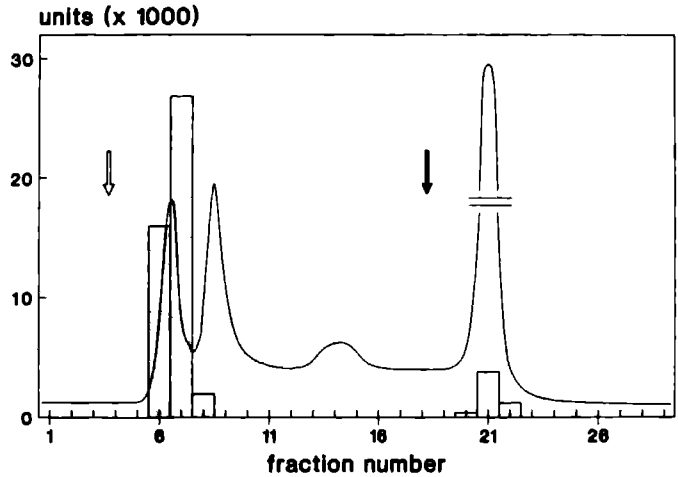

Figure 2. Chromatofocusing of concentrated urine. PBE 94 was used, eluated with 0.025 M ethanolamine (pH 9.4). One milliliter of sample was applied (*open arrow*), after which the column was regenerated with 1 M NaCl (*solid arrow*). Anti-elastase activity was measured in all fractions and expressed as units per fraction (*bars*). As can be seen from the elution profile (*solid line*; absorbance 280 nm; arbitrary scale) most of this anti-elastase activity elutes in the void volume, containing the material that does not bind to the column.

In the fractions collected after regeneration of the column with 1 M NaCl, only a small amount (less than 5%) of inhibitor was eluted. Determination of the dissociation constant K_i was carried out with the pooled fractions of the void volume of the chromatofocusing experiment, in which the highest anti-elastase activity was measured. Figure 3 shows an Easson-Stedman plot. The slope of the lines obtained for SKALP from urine and scales yields a similar K_i of approximately $2\text{-}10^{-11}$ M, which is in accordance with previous findings [12,13].

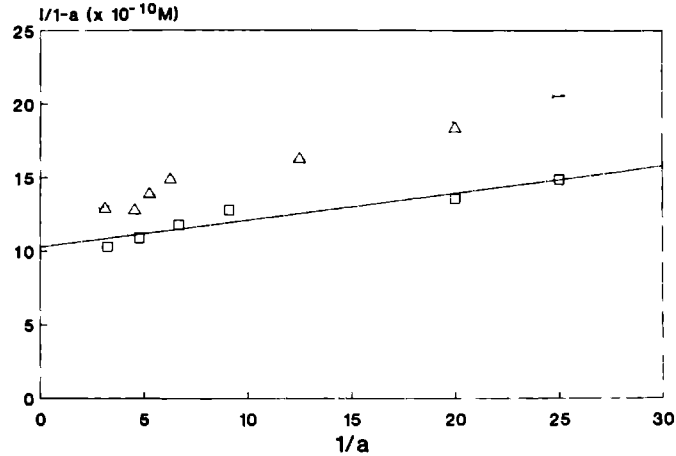

Figure 3. K_i determination of SKALP. Easson-Stedman plot of 1/a (a = fractional activity) against I/(1-a) (I = inhibitor concentration). See also *Materials and Methods* section. The slope of the line yields the apparent K_i. *Open triangles*: partially purified SKALP from urine, *open squares*: SKALP purified from scales.

SDS-PAGE and Western Blotting

The partially purified inhibitor preparation was subjected to SDS-PAGE. The gel was cut in 2-mm slices and assayed for anti-elastase activity as can be seen in Fig 4. Only one peak of activity was found, corresponding with a molecular weight of about 11 kDa, a value that was previously found for SKALP purified from psoriatic scales. In addition, gels were blotted on PVDF-membranes, which were used for protein staining and for immunostaining. As shown in Fig 5, the partially purified preparation obtained by chromatofocusing contains several low molecular weight bands, one of which coincides with the band of anti-elastase activity found in gel slices (see Fig 4). Using a polyclonal antiserum raised against highly purified SKALP from psoriatic scales, two bands were stained. The high molecular weight band coincides with the 11-kDa band of anti-elastase activity, the low molecular weight band probably represents an inactive breakdown product. A polyclonal antiserum against ALP/SLPI that is a related proteinase inhibitor with similar properties was tested in parallel. Both the anti-ALP/SLPI antiserum and preimmune rabbit serum (control for anti-SKALP) were

negative.

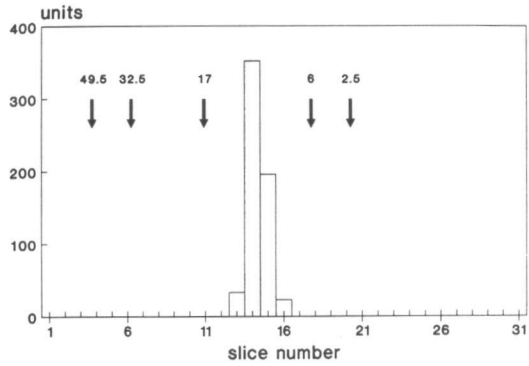

Figure 4. Anti-elastase activity eluted from an SDS gel. Partially purified urinary elastase inhibitor was electrophoresed under non-reducing conditions. The SDS gel was cut in 2 mm-slices and extracted in CTAB. The amount of anti-elastase activity recovered from the gel slices is given in units. The positions of marker proteins are indicated with *arrows* (kDa).

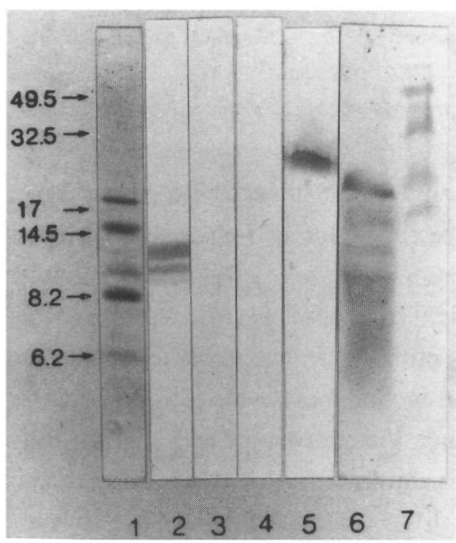

Figure 5. Western blotting of partially purified urinary elastase inhibitor. In *lane 2* distinct bands were stained with a polyclonal rabbit antiserum raised against SKALP from psoriatic scales. Preimmune serum (*lane 3*) and a rabbit polyclonal antiserum against ALP/SLPI (*lane 4*) were used as a control, the latter to exclude ALP/SLPI as the cause of the anti-elastase properties of the urine; both *lane 3* and *4* were negative. A positive control (10 ng recombinant ALP/SLPI) for the rabbit polyclonal antiserum against ALP/SLPI is shown in *lane 5*. Total protein staining of the urinary SKALP sample with amidoblack is shown in *lane 6*, and molecular weight markers (kDa) in *lanes 1* and *7*.

DISCUSSION

The structural integrity of human skin is at risk during acute inflammatory reactions when activated phagocytes (PMN, monocytes,

macrophages) invade the papillary dermis and epidermis, e.g., in psoriasis [21]. These inflammatory cells are known to secrete large amounts of proteinases that can damage extracellular matrix proteins, basement membranes, and desmosomal structures [1,9,22]. Normal human epidermis contains very little inhibiting activity against elastase, one of the major PMN-derived proteinases. However, we have recently shown that in a number of inflammatory skin diseases large amounts of epidermal anti-elastase activity can be found, in particular in psoriasis [12]. The major source of the anti-elastase activity could be ascribed to a cationic, low molecular weight protein that was given the acronym SKALP [13]. In view of its physico-chemical properties, SKALP will easily cross the basal lamina, penetrate the connective tissue, and reach the circulation. Because small cationic proteins are readily excreted in the urine, we investigated whether SKALP could be found in urine from psoriatic patients. In this paper we show that considerable amounts of anti-elastase activity can be found in urine from most of the psoriatic patients, whereas urine from healthy controls is usually negative. The anti-elastase activity could be ascribed to SKALP, based on biochemical and immunological criteria. The urinary anti-elastase activity as measured in our assay was not due to α1-proteinase inhibitor (data not shown). In addition we could exclude that the activity was due to ALP/SLPI, which is a proteinase inhibitor that shows structural and functional similarity to SKALP.

Interestingly, in some of the patients we found low levels of free elastase activity in the urine, whereas SKALP activity was undetectable. SKALP activity could be unmasked by boiling the urine, which destroys elastase activity, as shown in Fig 1. This finding emphasizes the importance of SKALP as a physiologically relevant elastase inhibitor.

The presence of SKALP in urine strongly suggests (but does not prove) that it is from epidermal origin. Immunohistological and functional studies indicate that the keratinocytes are the source of epidermnal SKALP; inflammatory cells were found to be negative (data not shown). Unfortunately we have no assay at the moment that allows quantification of immunoreactive SKALP. Once a SKALP-specific

enzyme-linked immunosorbent assay is available, the presence in plasma could be established, to link the presence in epidermis and urine. This would, in addition, allow a more sensitive measurement of SKALP and its breakdown products in urine. Preliminary results of measurement of biologically active SKALP in urine suggest a relationship between disease severity and urinary SKALP levels.

Future research will be aimed at developing sensitive, discriminative assays for detection of urinary SKALP (active and inactive fragments). This might yield an useful, noninvasive, quantitative measure for disease activity.

TABLE I

Anti-elastase activity in urine of psoriatic patients (n = 33) and controls (n = 10) in units SKALP per ml. Inhibitory activity was measured as described in the Materials and Methods section. Values below the detection limit of 200 units per ml urine are marked with nd (non detectable). A statistically significant difference in inhibitory activity between psoriatics and controls was found ($p < 0.002$, Mann-Whitney U-test).

Psoriatics								Controls			
	Sex	Age	SKALP		Sex	Age	SKALP		Sex	Age	SKALP
1.	f	66	880	18.	f	34	1408	1.	m	31	nd
2.	f	26	1568	19.	m	60	nd	2.	m	28	nd
3.	m	49	6656	20.	m	29	212	3.	m	36	nd
4.	m	40	816	21.	f	47	752	4.	m	28	nd
5.	m	42	nd	22.	m	65	768	5.	m	58	nd
6.	m	66	456	23.	f	67	232	6.	f	25	432
7.	m	35	416	24.	f	31	312	7.	m	44	nd
8.	m	38	nd	25.	f	77	nd	8.	m	33	nd
9.	m	50	352	26.	f	80	376	9.	f	36	nd
10.	f	86	668	27.	m	67	nd	10.	f	57	nd
11.	m	49	nd	28.	m	76	424				
12.	m	23	704	29.	f	36	232				
13.	f	68	424	30.	f	27	1856				
14.	f	23	nd	31.	f	24	1376				
15.	f	24	416	32.	f	18	456				
16.	m	18	288	33.	m	27	400				
17.	f	32	204								

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Chapter 7

Levels of skin-derived antileukoproteinase (SKALP)/elafin in serum correlate with disease activity during treatment of severe psoriasis with cyclosporin A

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ABSTRACT

The epidermal serine proteinase inhibitor SKALP (also known as elafin), directed against human leukocyte elastase and proteinase 3, is strongly induced in suprabasal keratinocytes during inflammation. The presence of SKALP/elafin in urine has been demonstrated for several inflammatory skin disorders, such as psoriasis, erythroderma and erysipelas. In this study we investigated whether SKALP/elafin levels in serum and urine of psoriatic patients can be used as a marker for disease activity during treatment. Patients with severe chronic disabling psoriasis were treated for 16 weeks with cyclosporin A, which resulted in a marked clinical improvement as measured with the PASI score. SKALP/elafin levels both in serum and urine were determined with an enzyme-linked immunosorbent assay (ELISA). Measurements were performed at the start of the cyclosporin A treatment, and after regular intervals up to 16 weeks. The results indicate that:

1. SKALP/elafin determination in serum rather than in urine is the prefered method, because the decrease in serum SKALP levels during therapy is more pronounced and correlated better with the clinical course of the patients.
2. SKALP/elafin levels in serum decreased during cyclosporin A treatment ($p<0.05$).
3. SKALP/elafin levels in serum correlate with the PASI score ($p<0.01$).

We conclude that SKALP/elafin measurement in serum of patients with severe psoriasis provides a tool for monitoring disease activity.

INTRODUCTION

We have recently shown that skin-derived antileukoproteinase (SKALP), a serine proteinase inhibitor which is virtually absent in normal epidermis, is induced in lesional psoriatic skin [1]. We found that SKALP, also known as elafin [2,3], is not specific for psoriasis, but can be considered as one of the markers of the regenerative differentiation

program that is also found in other conditions such as wound healing [4,5] and several inflammatory skin diseases [6]. SKALP/elafin is a strong inhibitor of the leukocytic enzymes elastase [1] and proteinase-3 [7] and is putatively involved in the regulation of cutaneous inflammation. In previous studies SKALP/elafin was described biochemically [8] and characterized at the protein and DNA level [9]. We established the cellular source and localization in psoriatic epidermis [10], and we found that SKALP/elafin is differentially expressed in human epidermal tumors [11]. Recently we have been able to assign the SKALP/elafin gene to chromosome region 20q12-q13 [12]. The gene has been given the approved name of Protease Inhibitor, skin derived (SKALP), symbol: PI3, in the Genome Data Base of the HUGO nomenclature committee.

In psoriasis, SKALP/elafin is expressed in the upper spinous layers of lesional skin [10]. We showed that SKALP/elafin as expressed in cultured epidermal keratinocytes is translated as a 12.3 kDa protein. Cleavage of the signal peptide yields a 9.9 kDa protein that is the major form found in cultured cells, as was confirmed by purification and N-terminal amino acid sequencing [9]. The presence of a signal peptide, suggests that SKALP/elafin is a secreted protein. We indeed found that significant amounts of biologically active SKALP/elafin can be found in the urine of psoriatic patients [13], a finding that was recently confirmed by others [14]. In urine of healthy individuals SKALP/elafin was not detectable. Apparently, once secreted in the epidermis, SKALP/elafin is able to reach the circulation, and is subsequently cleared via the kidney. Although we were then not able to measure SKALP/elafin in serum, this was a likely route, since SKALP/elafin is a low molecular weight cationic protein which will easily cross basal membranes.

In the present study there were two objectives: first, we wanted to develop an assay for SKALP/elafin assessment in serum of psoriatic patients, and second, we wanted to investigate if measurement of SKALP/elafin in urine and serum could be used as a marker for disease activity during therapy. In our previous study, we used a functional biochemical assay to measure SKALP/elafin in urine [13]. However, in

serum this is not possible due to interfering factors. We therefore developed an enzyme-linked immunosorbent assay (ELISA) which allows measurement of SKALP/elafin both in serum and in urine. Using this ELISA we found that patients with severe psoriasis during therapy with cyclosporin A (CyA) showed a decrease of SKALP/elafin activity in serum which correlated with a decrease in clinical scores (Psoriasis Area and Severity Index (PASI) score). These findings suggest that SKALP/elafin can be used as a marker for disease activity and can be used as a quantitative measure to monitor therapeutic effects.

MATERIALS AND METHODS

Chemicals

Tween-20 was obtained from Merck, Schuchardt, Hohenbrunn bei Munich, Germany, and bovine serum albumin (BSA) from Organon Teknika, Boxtel, The Netherlands. Swine-anti-rabbit peroxidase was obtained from Dako, Copenhagen, Denmark, and o-phenylene diamine.2HCl (OPD) from Pierce, Rockford, IL, USA. Microtiter plates (96 wells) for ELISA were obtained from Greiner, Alphen a/d Rijn, The Netherlands. Recombinant SKALP/elafin was a kind gift from Dr. Norman Russell, ICI Pharmaceuticals, UK.

Subjects, sample collection and preparation

Fifteen healthy volunteers (5 males, 10 females, age 2-79 years) took part in the experiments to study age or sex differencess in SKALP/elafin serum levels. For determination of circadian variation blood was drawn every 4 hours during 24 hours in a psoriatic patient and a healthy control. Six male patients (age 36-64 years, mean age 48 years) with severe chronic disabling psoriasis participated. Patients had not used any systemic treatment for at least 4 weeks or local treatment for at least 1 week prior to the start of CyA therapy. In all patients, initial dosage of CyA was 3 mg/kg body weight; in 2 patients this dosage was increased up to 4 mg/kg body weight after 8 weeks of treatment. Before treatment disease activity was evaluated and expressed using the PASI score. This

scoring system estimates disease activity depending on erythema, infiltration, desquamation, and the area of the psoriatic skin that is affected [15]. At the start of CyA therapy, and after 2, 4, 8, 12, and 16 weeks, samples of blood and urine were taken, and a PASI score as measure of disease activity. Samples of blood and urine were frozen, and stored at -20 °C until measurement of SKALP/elafin.

Anti-SKALP/elafin serum

Recombinant SKALP/elafin was used for immunization procedures as described before [8]. In short, a rabbit was immunized intracutaneously with recombinant SKALP/elafin that was crosslinked with glutaraldehyde and emulsified in Freunds complete adjuvant. A booster with the same preparation was given after 2 weeks, and 4 weeks later serum was collected via standard methods. Control (preimmune) serum was drawn before the immunization procedure. The specificity of the antiserum was validated as described before [8].

Enzyme-linked immunosorbent assay (ELISA)

To measure SKALP/elafin concentrations in serum and urine of psoriatic patients, we used a competitive-type ELISA.

Blood samples were taken, coagulated for 60 min at room temperature, and centrifuged at 450 g for 10 min. Serum was acidified to a final concentration of 0.05 N HCl, boiled for 2 min, and centrifuged for 60 min (2,000 g, 4 °C). Urine was boiled for 2 min, cooled on ice for 30 min, and centrifuged for 15 min (2,000 g, 4 °C). Creatinine measurement was done before by an alkaline picrate determination with kinetic endpoint detection, carried out with reagents from Boehringer Mannheim using a Hitachi 747 analyzer. Supernatants were taken for quantification of SKALP/elafin.

The supernatants were mixed to contain 80% supernatant, 0.1 M Tris, 0.1% Tween-20, 1% BSA, and rabbit anti-SKALP/elafin antiserum (1:9500 diluted), and incubated overnight. Microtiter plates (96 flat bottom wells) were coated overnight with 50 ng/ml recombinant SKALP/elafin in phosphate-buffered saline (PBS). After washing of the

plates with PBS/0.05% BSA, microtiter plates were blocked, probed with the test samples, and developed during 60 min with swine-anti-rabbit peroxidase using OPD as chromogenic substrate for 30 min. Human recombinant SKALP/elafin in PBS with 0.1% BSA was used as a standard: a calibration curve was made, using recombinant SKALP/elafin in the range of 0.6-80 ng/ml. The SKALP/elafin concentrations in serum or urine samples were read from this curve. All ELISA steps were performed at 4 °C, except development with OPD, which was done at room temperature. Data were read with a Biorad™ ELISA-reader, and evaluated using the Excell™ spread sheet program.

Statistical analysis

Regression analysis was performed to correlate SKALP/elafin levels in serum and urine with each other, with time of CyA treatment, and with the disease activity as expressed in the PASI score. Mean r (Pearson moment product correlation) was obtained after Fisher-z transformation.

RESULTS

PASI score and CyA treatment

All patients showed good clinical improvement during 16 weeks of treatment with CyA, and apart from slight gastrointestinal discomfort no complaints were reported. Mean PASI score was 20.0 (10.6-36.0) at the start of the treatment, and decreased to 3.4 (2.0-5.4) at week 16. In 2 patients dosage of CyA were increased at week 8 due to lack of clinical progress. Using routine blood tests for liver and kidney functions no side effects on these organs could be demonstrated; one patient showed hypertrichosis. Urine and blood samples of one of the patients could not be taken until 2 weeks after the start of the CyA treatment.

Sensitivity and accuracy of the ELISA

The ELISA we developed is of the competitive type, based on the displacement of a defined amount of recombinant SKALP/elafin by SKALP/elafin in the urine or serum samples. A calibration curve from

which the sample values are read in the interval between 3-40 ng per ml, is given in figure 1. The detection limit is 3 ng/ml.

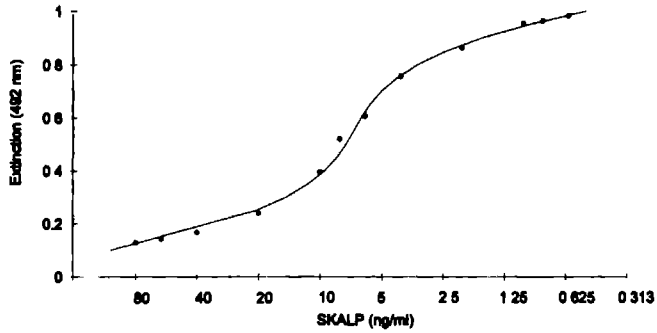

Figure 1. Calibration curve of recombinant SKALP/elafin. A sigmoid relationship between the ELISA signal and the SKALP/elafin concentration of the samples is found. The useful range for measurement of SKALP/elafin concentrations is between 3-40 ng/ml.

Experiments to check the recovery of recombinant SKALP/elafin added to normal human serum revealed that recovery is temperature dependent (not shown). When the ELISA was performed at 4 °C a recovery of 100% was found (figure 2).

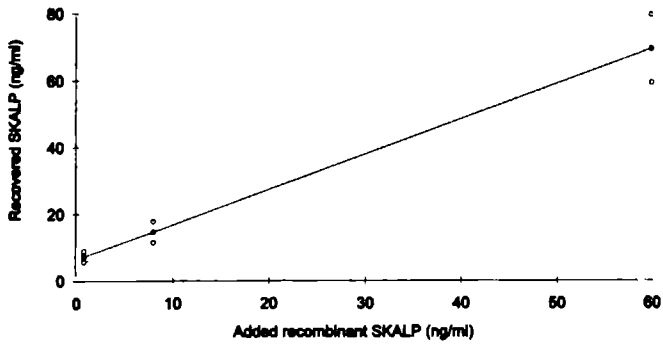

Figure 2. Mean values ± 2 standard deviations of measurements in quadruplicate of 3 concentrations of recombinant SKALP/elafin in normal human serum. Standard deviation is 10%. The intercept with the Y-axis indicates that in humans a background serum level of SKALP/elafin appears to be present. This background level is about 9 ng/ml.

Figure 2 also shows that a pool of normal human serum has a background level of SKALP/elafin per ml, which appeared to be about 9 ng/ml (intercept of the Y-axis). The standard deviation of measurements in quadruplicate was within 10%.

SKALP/elafin measurement in urine

SKALP/elafin levels were expressed in ng/ml urine, and corrections for creatinine concentrations were made. SKALP/elafin levels in urine varied from 3 ng/ml up to more than 120 ng/ml (Figure 3). Five out of six patients showed a clear decrease in urinary SKALP/elafin levels. One patient showed extremely low values of creatinine concentration, which had an enormous impact on correction for creatinine (Figure 3c); note that his serum level of SKALP/elafin decreased during treatment (Figure 4c). Decrease of SKALP/elafin levels in urine during treatment was statistically significant (Fisher-z, $p<0.05$).

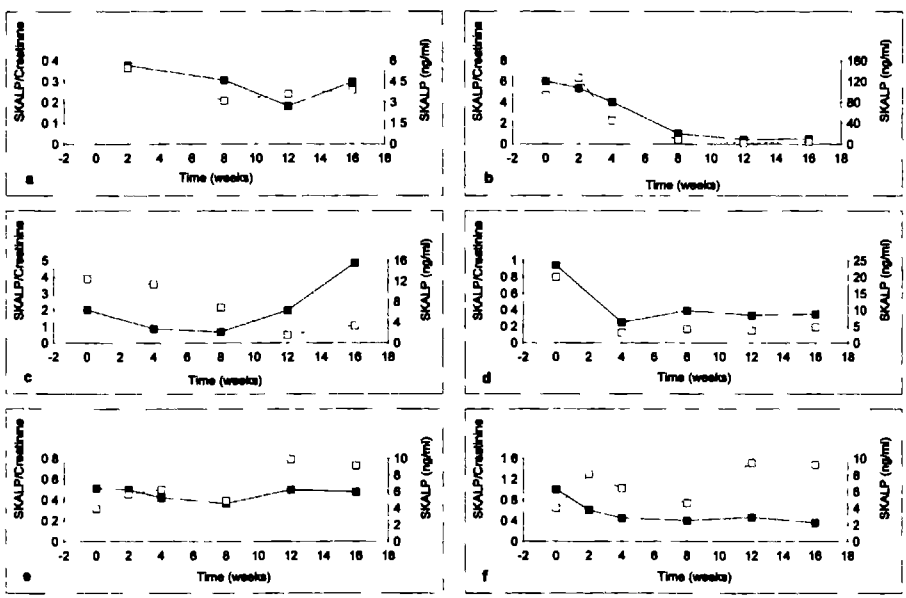

Figure 3. SKALP/elafin levels in urine of 6 psoriatic patients (a-f) during treatment with CyA. SKALP/elafin levels (ng/ml) are shown before (open squares) and after correction for creatinine concentration (solid squares). Decrease of SKALP/elafin levels in urine is statistically significant (Fisher-z, $p<0.05$).

Urinary SKALP/elafin levels decreased, but were not found below 3 ng/ml. Healthy controls do show a similar amount of SKALP/elafin in urine (data not shown), and 3-10 ng/ml may be regarded as a normal background level of SKALP/elafin in human urine.

SKALP/elafin measurement in serum

Serum levels for SKALP/elafin were measured in 15 healthy volunteers. No effect of age on SKALP/elafin levels was found (not shown). A small difference in SKALP/elafin serum levels (ng/ml) was found between males (14.6±2.6) and females (8.5±6.1). No circadian rhythm was observed (not shown). SKALP/elafin levels psoriatic patients varied from 11 ng/ml up to more than 300 ng/ml (Figure 4).

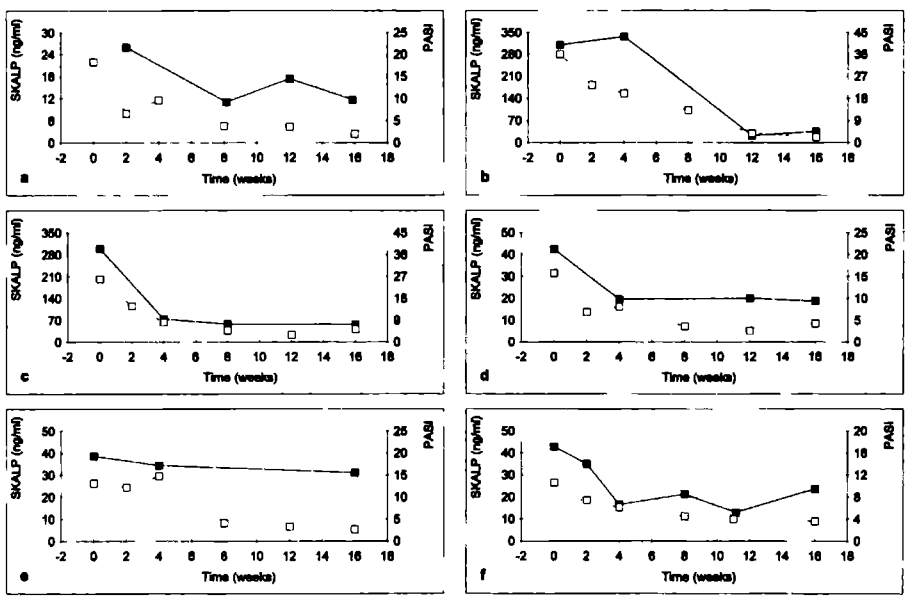

Figure 4. SKALP/elafin levels in serum of 6 psoriatic patients (a-f) during treatment with CyA. Amount of SKALP/elafin in serum is given in ng/ml (solid squares). Decrease of SKALP/elafin levels in serum is statistically significant (Fisher-z, $p<0.05$). PASI scores at time of sampling are given (open squares).

These levels significantly decreased during treatment with CyA (Fisher-z, $p<0.05$) and correlated well with clinical improvement as expressed in

the PASI score (Fisher-z, $p<0.01$). This decrease in SKALP/elafin level was found in the serum of all six patients. SKALP/elafin levels in serum were about sixfold higher than in urine, and levels in serum were positively correlated with levels in urine (Fisher-z, $p<0.01$).

DISCUSSION

Increased amounts of SKALP/elafin have been demonstrated in urine of patients with psoriasis [13] and several other inflammatory skin diseases [14]. Since SKALP/elafin is barely detectable in normal skin and is strongly expressed in inflamed skin, we hypothesized that the increase in urinary SKALP/elafin in psoriatics is derived from local production in skin. This would be in line with the fact that SKALP/elafin is a secreted molecule [16] and the notion that epidermis acts as a secretory organ as recently described [17,18]. However, no definitive proof could be given for the epidermal origin of urinary SKALP/elafin in psoriatic patients, because SKALP/elafin could not be demonstrated in the intermediate compartments between epidermis and urine (i.e. dermis and blood). In our initial study we used a functional assay to measure SKALP/elafin, that could not be applied to serum, due to interference by the overwhelming amount of other plasma-derived proteinase inhibitors.

Intraepidermal accumulation of PMN is an early event in the pathogenesis of the psoriatic lesion [19], and PMN derived proteinases were shown to degrade the dermo-epidermal junction in vitro [20]. This suggests that elastase may be involved in PMN migration and PMN dependent tissue damage and may promote the inflammatory response. Several antipsoriatic therapies are known to interfere with this PMN migration, suggesting that they are involved in the downregulation of inflammation [21]. In addition to proteinases from inflammatory cells, keratinocyte derived proteinases such as plasminogen activator may act as a proinflammatory mediator in psoriatic skin [22]. Synthesis of proteinase inhibitors that counteract proteinases by forming inactive complexes is one of the *in vivo* mechanisms to protect tissue against unwanted proteolysis [23]. These proteinase inhibitors may be present in

plasma, or can be produced locally at the site of action [8,24,25]. Complexes between elastase and elastase inhibitors in serum, sputum, bronchoalveolar lavage, and urine, have been described, the inhibitors being α1-PI [26-28], antileukoproteinase [29] or SKALP/elafin [13]. Levels of these complexes may be related to disease activity, and measurement may provide a tool for monitoring therapeutic result. Increased levels of complexes between elastase and inhibitors, but also of free elastase or free inhibitor have been reported in lung cancer [30], neonatal systemic infection [31,32], adult respiratory distress syndrome [27,32], cervical cancer [33,34], psoriasis, eczema and other cutaneous disorders [35-37], and these levels can be used as parameters for monotoring disease activity.

In normal epidermis anti-elastase activity is hardly detectable [1], although the presence of α1-PI, and α2-macroglobulin in normal skin has been described [38,39]. Teleologically, it is plausible that after a disturbance of the proteinase-antiproteinase balance in skin, resulting in an excess of elastase, SKALP/elafin is produced to limit proteolytic action of this leukocytic proteinase. After a period of proteolysis by elastase a rebalancing takes place, and SKALP/elafin molecules may temporarily outnumber elastase molecules. Complexes are secreted in urine [13], but epidermic clearance via loss of scales that contain complexes may take place as well. Finally, restoration of normal homeostasis is followed by an off-switch of SKALP/elafin production by the epidermal cells.

Using a competitive-type ELISA, we were able to demonstrate the presence of SKALP/elafin in serum of normal individuals and psoriatic patients. The assay was found to be reproducable and reasonably sensitive (detection limit 3 ng/ml). We found a background level of SKALP/elafin in serum of healthy controls and symptom-free psoriatic patients which is probably derived from the turnover in tissues where SKALP/elafin is constitutively expressed. We have recently completed a survey of SKALP/elafin in normal human epithelia (Alkemade *et al.*, manuscript in preparation) and found that the molecule is expressed in a limited number of normal tissues. This background level of

SKALP/elafin in serum negatively influences the sensitivity of the assay for detecting an increase in inflammatory skin diseases.

In severe psoriatic patients with a large area of involved skin, serum levels are significantly higher than in healthy controls and symptom-free psoriatic patients. It is shown that during therapy with CyA, which is a potent antipsoriatic drug, serum levels gradually decrease and stabilize to a level which is still higher than in normal controls. This decrease in SKALP/elafin levels correlate with the decrease in PASI scores, and we assume that the decrease in serum SKALP/elafin levels during therapy reflects the rate of normalization of the epidermis. The SKALP/elafin concentrations in serum were about sixfold higher than in urine, and the decrease in serum during treatment was more impressive and correlated better with the clinical course. Therefore, determination of SKALP/elafin in serum rather than in urine was considered to be the method of preference. Because SKALP/elafin levels correlated well with the clinical state as represented by the PASI score, we conclude that SKALP/elafin measurement in serum of patients with severe psoriasis provides a tool for monitoring disease activity.

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Chapter 8

Differential expression of SKALP/elafin in human epidermal tumors

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ABSTRACT

Recently we described a new epidermal serine proteinase inhibitor, skin-derived antileukoproteinase (SKALP), also known as elafin. SKALP/Elafin was found to be absent in normal human epidermis, but can be induced *in vitro* and *in vivo* under hyperproliferative conditions. Here we studied the expression of SKALP/elafin in several types of epidermal tumors (basal cell carcinoma, squamous cell carcinoma, Bowen's disease, actinic keratosis, and keratoacanthoma). Using immunohistochemical staining SKALP/elafin appeared to be differentially expressed in these tumors. Functional measurements of antiproteinase activity, and Western blotting of tumor extracts confirmed our findings at the histological level. In well-differentiated squamous cell carcinoma, SKALP/elafin messenger RNA was demonstrated by non-radioactive *in situ* hybridization. We conclude that SKALP/elafin is a marker for abnormal or disturbed squamous differentiation. A possible role of SKALP/elafin in the control of tumor cell invasion is discussed.

INTRODUCTION

Deregulation of gene expression may cause transcription of oncogenes or loss of action of growth suppressor genes, leading to abnormal growth of tumor cells. However, in addition to disturbed growth control, also invasion and metastasis are features of malignant behavior. Several proteinases such as plasminogen activators, cathepsins and metalloproteinases, as well as the inhibitors of these enzymes have been reported to be associated with tumor invasion and metastasis [1-9]. Expression of proteinases is thought to promote the migrational capacity of cells; specific inhibitors of these proteinases counteract this process. Hence, the term metastasis suppressor proteins has been coined for these inhibitors [10,11].

Recently we described a new serine proteinase inhibitor (skin-derived antileukoproteinase, SKALP) which is expressed in lesional psoriatic epidermis and in epidermis after injury, but is absent in normal

epidermis [12,13]. Initially, SKALP was shown to be a low molecular weight, cationic, heat-stable protein, that was similar to elafin, an epidermal proteinase inhibitor described by others [14]. We have recently cloned and sequenced the cDNA of SKALP, which proved SKALP and elafin to be identical, and which showed that SKALP/elafin as expressed in cultured epidermal keratinocytes is translated as a 12.3-kd protein [15]. Cleavage of the signal peptide yields a 9.9-kd protein that is the major form found in cultured cells, as was confirmed by purification and N-terminal amino acid sequencing [15]. Elastase-specific inhibitors from bronchial secretions with molecular weights of 10 kd, 5 kd, and 2.5 kd as reported by Hochstrasser, Kramps and Sallenave respectively [16-18], are biological active fragments of the same larger precursor molecule [19]. SKALP/Elafin inhibits at least three serine proteinases derived from polymorphonuclear leukocytes (PMN), namely human leukocyte elastase, porcine pancreatic elastase, and human leukocyte proteinase 3 [12,13,20].

Recently we could assign the chromosomal localization of the SKALP gene to chromosome 20, band q12-13 [21]. The gene has been given the approved name of Proteinase Inhibitor, skin derived (SKALP), symbol: PI3, in the Genome Data Base of the HUGO nomenclature committee.

The exact biological function of SKALP/elafin is not known at present, although the substrate specificity for polymorphonuclear cell-derived, elastolytic proteinases suggests that SKALP/elafin is involved in regulation of cutaneous inflammation, or protection against polymorphonuclear cell-dependent tissue damage. However, it is very likely that other target enzymes (eg, from the keratinocytes or dermal fibroblasts) exist; hence other functions cannot be excluded.

Because the expression of SKALP/elafin appears not to be associated with the differentiation program of normal epidermis but is found in differentiating cells in the context of the hyperproliferative differentiation program (eg, psoriasis and wound healing), we investigated its expression in a number of epidermal tumors that are characterized by abnormal differentiation. The data clearly show that SKALP/elafin is differentially expressed in these tumors.

MATERIALS AND METHODS

Chemicals

Methoxysuccinyl-alanyl-alanyl-prolyl-valyl-7-amino-4-methyl coumarin, was obtained from Bachem, Bubendorf, Switzerland. Cetyltrimethyl ammoniumbromide was obtained from ICI, UK. All reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) including prestained markers were obtained from Bio-Rad Laboratories, Richmond, CA. Polybuffer exchanger 118, a Superdex 75 fast protein liquid chromatography column, a Superdex 75 PC 3.2/30 column, and a SMART chromatography system were obtained from Pharmacia, Uppsala, Sweden. Polyvinylidenedifluoride membrane was from Millipore, Etten-Leur, the Netherlands. Low molecular weight markers used for SDS gels, goat anti-rabbit IgG biotin conjugate, avidin-alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate, and nitroblue tetrazolium were obtained from Sigma Chemicals, St. Louis, MO. Calibration proteins used in chromatography were obtained from Boehringer Mannheim GmbH, Mannheim, Germany. Recombinant elafin was a gift from Dr. Norman Russell, ICI Pharmaceuticals, United Kingdom.

Tumors

Material used for histology was selected from archive biopsy material sent for routine analysis. The specimens had been fixed in buffered 4% formalin for at least 24 hours, and had been embedded in paraffin. Seven basal cell carcinomas, seven squamous cell carcinomas, three Bowen's diseases, seven actinic keratoses, and seven keratoacanthomas were selected. The tissue was cut in 5-μ sections, using a Reichert Jung rotation microtome (Leica, Rijswijk, The Netherlands), and mounted on slides, which were coated with 3-aminopropyltriethoxysilane. Sections were dried 48 hours at 37 °C. Some of the sections were used for *in situ* hybridization experiments as well.

A limited amount of fresh tumor material was available for extraction and was used for functional measurements and Western blotting (two

basal cell carcinomas, two keratoacanthomas and one squamous cell carcinoma).

Construction of plasmid and synthesis of RNA probes

A 0.42-kb *Pvu*II/*Eco*RI fragment of pGESKA [15], containing almost complete SKALP/elafin complementary DNA was cloned into the plasmid pGEM4 (Promega, Madison, WI). Sense and anti-sense complementary RNA probes were prepared. Briefly, after linearizing the construct with either *Bam*HI or *Kpn*I, the anti-sense and sense cRNAs were transcribed *in vitro* using digoxigenin-labelled UTP, T7 and SP6 RNA polymerase, respectively, according to the manufacturer's instructions (Boehringer). The cRNAs were fragmented with limited alkaline hydrolysis to reduce the size of the synthesized RNAs to approximately 0.2 kb [22].

***In situ* hybridization**

Nonradioactive *in situ* hybridization was, with some modifications, performed as described by Yokouchi et al [23]. Sections were deparaffinized with xylene, rehydrated using solutions of diminishing ethanol concentrations (99.8 to 50%), and finally rinsed in phosphate-buffered saline (PBS). Sections were then treated with 0.01% Triton X-100 for 1.5 minutes, rinsed with PBS, incubated with pepsin 1 mg/ml in 0.2 N HCl for 15 minutes at 37 °C, and again rinsed with PBS. Thereafter postfixation with 4% paraformaldehyde in distilled water was performed. After acetylation with 0.1 M triethylamine, pH 8.0, and 0.25% acetic anhydride for 10 minutes, sections were rinsed with PBS and dehydrated with increasing concentrations of ethanol (50 to 99.8%).

The hybridization mixture consisted of 50% formamide, 2X standard sodium citrate (SSC), 10% dextransulfate, 5X Denhard's solution (0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400), 10 mM dithiothreitol, 1 mg/ml *Escherigia* coli transfer RNA, and 4 μg/ml probe. After overnight hybridization at 37 °C, the sections were washed twice for 45 minutes at 37 °C in 2X SSC and 1X SSC, respectively, incubated for 20 minutes at room temperature with 20 μg/ml DNase free

RNase A, (Boehringer) and 50 mM NaCl in Tris EDTA. A second wash step with 1X SSC was carried out for 30 minutes at room temperature.

Hybridization signals were detected by an enzyme-linked immunoassay using a nucleic acid detection kit as suggested by the manufacturer (Boehringer).

Isolation and purification of SKALP/elafin

Skin-derived antileukoproteinase/elafin was isolated both from psoriatic scales and from cultured human keratinocytes as described before [12,13]. In short, psoriatic scales were homogenized in distilled water, yielding a suspension that was boiled and centrifuged, followed by chloroform extraction and centrifugation. After concentration, the preparation was further purified by chromatofocusing (PBE 118 column, triethylamine buffer) and affinity chromatography (porcine pancreatic elastase coupled to cyanogen bromide-activated Sepharose 4B, phosphate-buffered saline (PBS) washing buffer, 0.1 M acetic acid eluting buffer). Final purification was by gel permeation chromatography on a Superdex-75 fast protein liquid chromatography column. Extracts of cultured keratinocytes were prepared by sonication of the cells in distilled water and subjected to the SMART chromatography system (Superdex 75 PC 3.2/30 column). Anti-elastase activity in obtained fractions was measured; relevant fractions were pooled and vacuum evaporated to dryness, dissolved in nonreducing SDS-sample buffer and used for SDS-PAGE.

Anti-SKALP/elafin serum

Skin-derived antileukoproteinase/elafin purified from psoriatic scales or recombinant elafin was used for immunization procedures as described before (13). In short, a rabbit was immunized intracutaneously with highly purified SKALP/elafin that was partially crosslinked with glutaraldehyde and emulsified in Freunds complete adjuvant. A booster with the same preparation was given after 2 weeks, and 4 weeks later serum was collected via standard methods. Control (preimmune) serum was drawn before the immunization procedure. The specificity of the

antiserum was validated on Western blots and in functional assays which showed 1) that the elastase-inhibiting activity could be absorbed by the antiserum and 2) that the band stained on a Western blot corresponded with a band of anti-elastase activity eluted from the SDS-PAGE gel as shown before [13]. The two antisera yielded identical staining patterns both in immunohistology and Western blotting.

Immunohistology

Before staining, sections were deparaffinized with xylene, rehydrated using solutions of diminishing ethanol concentrations (99.8 to 50%) and finally rinsed in PBS. Thereupon, sections were preincubated with normal swine serum 20% for 15 minutes, and incubated with a polyclonal rabbit antiserum against SKALP at a dilution of 1/100 in PBS with 1% bovine serum albumin for 60 minutes. After washing in PBS, sections were incubated with peroxidase conjugated swine anti-rabbit immunoglobulin at a dilution of 1/50 in PBS with 1% bovine serum albumin and 5% human AB serum. Subsequently, sections were washed in PBS and Na-acetate buffer and developed with aminoethylcarbazole as the chromogenic substrate (incubation period, 10 minutes). Finally, sections were washed in distilled water, counterstained with hematoxylin and eosin, washed again, dried and embedded in glycerol-gelatin solution.

Control staining was performed as indicated above, with polyclonal rabbit anti-SKALP/elafin serum substituted by preimmune serum of the same animal.

SDS-PAGE and Western blotting.

Three types of tumors were studied (basal cell carcinoma, squamous cell carcinoma, and keratoacanthoma). Tumor tissue (average weight approximately 110 mg) was homogenized in distilled water with the help of a glass-glass grinder, whereafter the homogenate was centrifuged for 10 minutes at 12,000 revolutions per minute. Anti-elastase activity of the supernatant was assayed as described before [12,24]. The clear supernatant was vacuum evaporated to dryness, and subsequently

dissolved in 500 µl nonreducing SDS-sample buffer; 5µl of this preparation was subjected to SDS-PAGE. As a reference, samples of recombinant elafin, psoriatic scale extract, and partially purified SKALP/elafin of cultured keratinocytes were used in the same run of electrophoresis. Proteins were separated on a 16% polyacrylamide gel, using tricine as a trailing ion instead of tris(hydroxymethyl) aminomethane-glycine [25]. Gels were blotted on polyvinylidene difluoride membranes, and immunologic detection of proteins was performed using biotinylated goat anti-rabbit immunoglobulin G, avidin-conjugated alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate, and nitroblue tetrazolium. Both a polyclonal rabbit antiserum raised against highly purified SKALP/elafin from psoriatic scales and a rabbit antiserum against recombinant elafin were used.

Microphotography

Microphotographs were taken with a Leitz photomicroscope and a Zeiss Axiophot, Wetzlar and Oberkochen/Württemberg, Germany, respectively, using black-and-white negative film Agfapan APX 25 from Agfa-Gevaert AG, Leverkusen, Germany.

RESULTS

Immunohistology

In basal cell carcinoma, all tumor cells with a typical basaloid phenotype were negative with respect to SKALP/elafin expression. Cells of the granular layer in epidermis overlying the tumor were positive in some cases. The epidermis of distant normal skin did not show any SKALP/elafin expression at all (not shown).

In squamous cell carcinoma, SKALP/elafin expression varied both between tumors and within the individual tumors. However, most tumors and tumor cell nests that had a clear squamous phenotype were strongly positive as shown in Figure 1. In all specimens basal cells were negative. SKALP/elafin expression was seen from the suprabasal cells upward, with the strongest staining in the cells just underneath the layer of the

cornified envelopes, namely, in the stratum granulosum and the most superficial layers of the stratum spinosum.

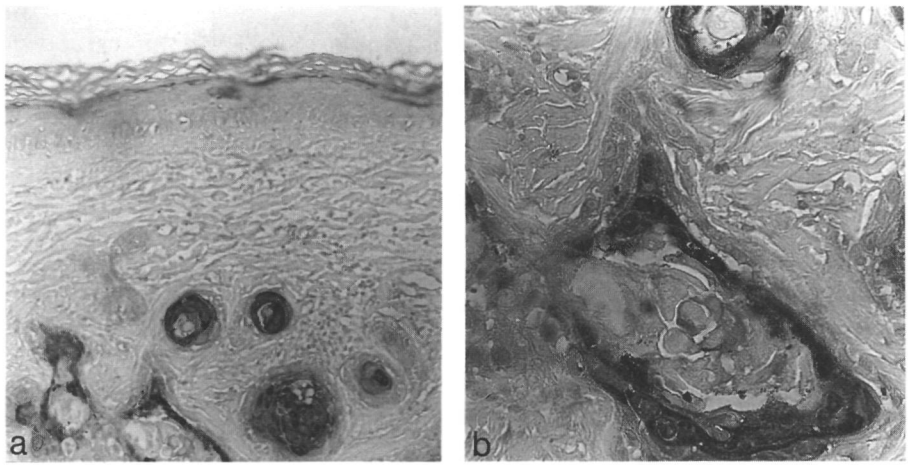

Figure 1. Immunohistology of squamous cell carcinoma, staining was performed with anti-SKALP/elafin antibody. (a) Cell nests with a clear squamous phenotype are strongly positive. Overlying epidermis is negative. Magnification: x125. (b) Detail. SKALP/elafin staining is most distinct in cells with a granular cell morphology. Magnification: x313.

Figure 2. Immunohistology of actinic keratosis. Variation in intensity of SKALP/elafin staining and in the number of stained cell layers as well, was observed. Here SKALP/elafin staining is limited to one or two layers of granular cells, just beneath ridges of thickened stratum corneum. Magnification: x125.

In Bowen's disease we found that the tumor cells showed a weak cytoplasmic staining, which was less pronounced than in the well differentiated squamous cell carcinomas (not shown).

All biopsies from actinic keratoses were positive, with a variation both

in intensity of SKALP/elafin expression and in the number of cell layers that were stained (two to eight layers, basal cells were negative; an example of an actinic keratosis with only one to two positive layers of keratinocytes is shown in Figure 2).

Keratoacanthomas were strongly positive for SKALP/elafin as shown in Figure 3.

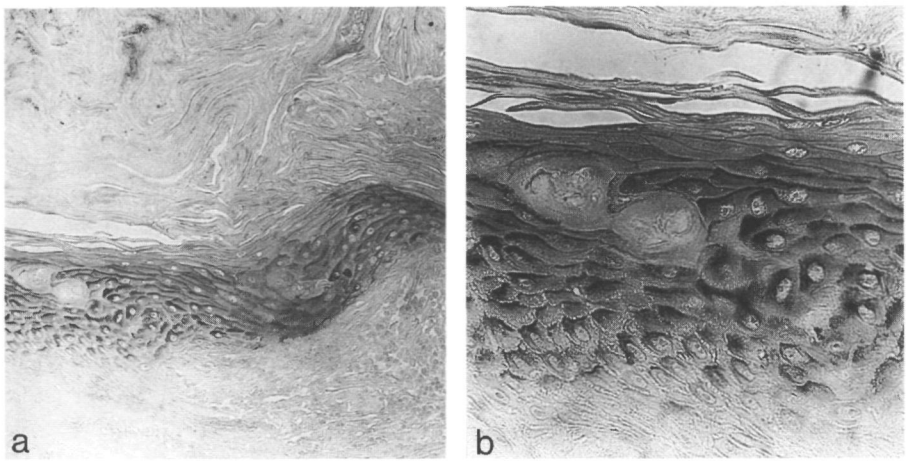

Figure 3. Immunohistology of keratoacanthoma. (a) Strong SKALP/elafin staining is seen in multiple layers of well differentiated suprabasal cells. Magnification: x125. (b) Detail. Note the polarized distribution of SKALP/elafin-staining pattern close to the upper cell membrane, in the direction of increasing differentiation, possibly because of cross-linking to the cornified envelope by transglutaminase. Magnification: x313.

The staining pattern was comparable to that of the other tumors, i.e., the suprabasal layers were stained, and staining was most pronounced in the cell layer just below cornified envelope formation.

Staining of the stratum corneum in all positive tumors was inconsistent, varying both in intensity and distribution pattern from strong to nearly absent, and from continuous to patchy.

In situ hybridization

Using cRNA probes, sections of a well differentiated squamous cell carcinoma, which showed high expression of SKALP/elafin using

immunohistochemistry, were studied for the presence of SKALP/elafin messenger RNA. In general, mRNA expression colocalized with expression of SKALP/elafin at the protein level as shown on serial sections (Figure 4).

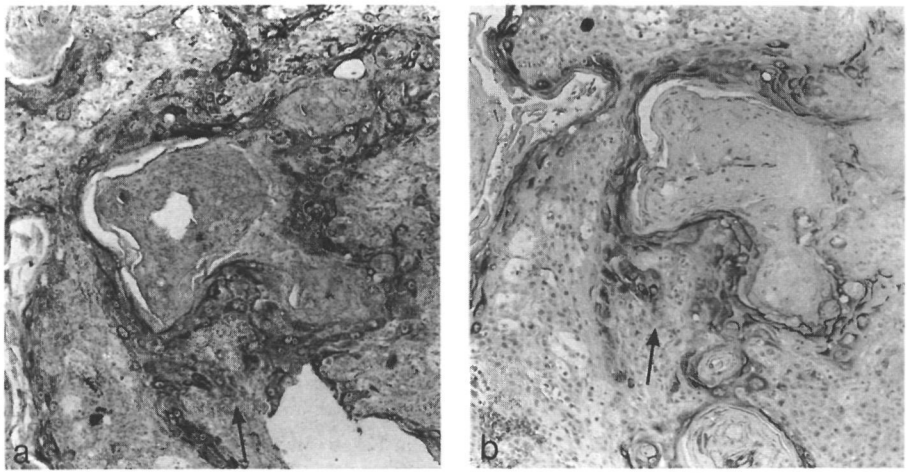

Figure 4. *In situ* hybridization and immunohistology on serial sections of a well differentiated squamous cell carcinoma. Using antisense cRNA probes, SKALP/elafin messenger RNA is shown (a), and immunostaining demonstrates presence of SKALP/elafin protein (b). Note that the expression at the mRNA level roughly coincides with the expression at the protein level; eg, see the region indicated by the arrows. Magnification: x100.

Sense cRNA probes, used as controls, were completely negative.

Functional measurements and Western blotting.

Using a sensitive microassay [12,24], both squamous cell carcinoma and keratoacanthoma showed inhibitory activity against human leukocyte elastase, whereas basal cell carcinoma was negative. Squamous cell carcinoma contained an anti-elastase activity of 2.3 U/mg tissue, anti-elastase activity of keratoacanthoma was 167 U/mg tissue, and in basal cell carcinoma no inhibitory activity was measured. Western blotting showed SKALP/elafin expression both in squamous cell carcinoma and in keratoacanthoma, but not in basal cell carcinoma (Figure 5).

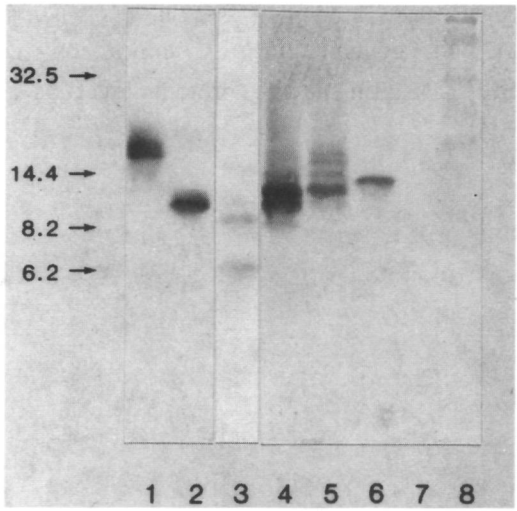

Figure 5. Western blot of human skin tumor extracts stained with a polyclonal anti-SKALP/elafin serum. In *lane 5* distinct bands were stained in a squamous cell carcinoma extract. In *lane 6*, only a single band is seen in an extract from keratoacanthoma. *Lane 7* shows the absence of SKALP/elafin staining in a basal cell carcinoma extract. Partially purified SKALP/elafin of cultured keratinocytes, recombinant elafin, and psoriatic scale extract have been used as reference samples, and staining is shown in *lanes 1*, *2*, and *4*, respectively. Molecular weight markers (kDa) are shown in *lanes 3* and *8*.

Interestingly, there were clear differences in staining patterns between the tumors. Keratoacanthoma showed one clear band with an apparent molecular weight of 13.6 kd. In contrast, squamous cell carcinoma showed four different bands with apparent molecular weights of 15.7 kd, 15.0 kd, 13.8 kd and 12.4 kd. Basal carcinoma was negative. SKALP/Elafin from cultured keratinocytes (apparent molecular weight about 16 kd), recombinant elafin (apparent approximate molecular weight 11 kd) and psoriatic scale extract were used as positive controls and as a reference range for the various forms of SKALP/elafin that are known to exist [12,13].

DISCUSSION

In this study we have demonstrated that the recently discovered serine

proteinase inhibitor SKALP/elafin is differentially expressed in human epidermal tumors. On immunohistology, the cytoplasmatic staining was mostly limited to the upper differentiating layers of the tumors. Basal layers were completely negative, which is in accordance with previous findings in psoriatic epidermis and in a model for wound healing [26,27]. Basal cell carcinomas were negative as well. *In situ* hybridization of well differentiated squamous cell carcinoma showed presence of messenger RNA in the same regions where SKALP/elafin protein is demonstrated by immunostaining.

Previous studies showed that SKALP/elafin exists in multiple forms [12-14]. In material from psoriatic patients a consistent pattern of two major bands emerges. As shown in Figure 5, in squamous cell carcinoma and keratoacanthoma different forms of SKALP/elafin are found. Because only a limited number of fresh tumors was available to us, it is not clear whether these patterns are specific for a type of tumor. The antisera used in this study were polyclonal rabbit antisera against recombinant elafin or SKALP/elafin purified from psoriatic scales. These sera yielded identical staining patterns on Western blots and in immunohistology. Both sera were raised against SKALP/elafin fragments that contained the anti-proteinase activity, which is located in the C-terminal half of the mature SKALP/elafin molecule. Because the different forms of SKALP/elafin are generated by various N-terminal deletions, all these cleavage products are recognized by these antisera. As can be seen in Figure 5, the apparent molecular weights of SKALP/elafin obtained on SDS-PAGE significantly deviate from the calculated molecular weights (as mentioned above). This is probably caused by the strong cationic nature of the molecule (isoelectric point = 9.6) which causes a slower migration in this electrophoresis system.

At present there are no conclusive data on the biological significance of SKALP/elafin in human skin. However, previous studies suggest that the expression of SKALP/elafin *in vivo* is linked to inflammatory processes. We have shown that SKALP/elafin-activity is found in epidermis from inflammatory skin diseases and that it is absent in normal skin and in non-inflammatory skin diseases [28]. Recently we

have found that SKALP/elafin is secreted in urine of psoriatic patients and that the levels found roughly correlate with the severity of the disease [29]. In addition we have found that SKALP/elafin can be induced in normal human skin as a result of standardized injury by tape stripping [12,27]. These findings show that SKALP/elafin is induced in human skin under inflammatory conditions, which suggests (but not proves) that SKALP/elafin could act as a negative feedback on the inflammatory response. Whether this would be at the level of interference with migration of polymorphonuclear cells/monocytes or as a protective mechanism against tissue damage remains to be investigated.

Apart from this teleological interpretation, SKALP/elafin expression can also be regarded as part of the regenerative/hyperproliferative differentiation program of human epidermis as seen in psoriasis and wound healing. The normal differentiation program includes the expression of keratin 1 and 10, the absence of keratin 6 and 16 in the suprabasal layers, and expression of differentiation-related proteins (eg, filaggrin, involucrin, transglutaminase) exclusively in the stratum granulosum [30]. The differentiation program of the regenerative/ hyperproliferative type is characterized by the induction of keratin 6 and 16, down-regulation of keratin 10, premature expression of differentiation-related proteins and, as we have shown, the induction of SKALP/elafin. Within the context of this differentiation program, SKALP/elafin could function as an inhibitor of proteinases from inflammatory cells or could, speculatively, be directed to keratinocyte-derived proteinases to control other, hitherto unknown processes. Evidence that SKALP/elafin expression is coupled to the regenerative/hyperproliferative phenotype is further supported by the notion that cultured keratinocytes, which are also negative for keratins 1/10 and positive for keratins 6/16, produce large amounts of SKALP/elafin [12,15].

In this study we demonstrate that SKALP/elafin expression is high in tumors with a clear squamous phenotype, and is absent in poorly differentiated squamous cell carcinomas and in basal cell carcinomas. Tumor cells display disturbed differentiation programs that are distinct

from the differentiation programs mentioned above. Speculatively, loss of expression of anti-proteinase activity could promote tumor cell migration, invasive growth, or induce detachment of tumor cells. This could be caused either by proteinase activity from the tumor cells or by proteinases derived from neighbouring cells (stroma, inflammatory cells). The finding in the present study that SKALP/elafin expression is low or absent in the tumors that are able to invade the dermal connective tissue is in line with this hypothesis.

In general, little is known with respect to the role of other proteinases and proteinase inhibitors in normal human skin. It has been suggested that cathepsin D is involved in transglutaminase processing [31]. Both urokinase-type plasminogen activator and its inhibitor, plasminogen activator inhibitor were demonstrated in human epidermis [32-34], and are supposed to be involved in keratinocyte migration [35,36]. Expression levels of proteinases and proteinase inhibitors have been reported to correlate with tumor proliferation, tissue invasion and tissue destruction. The resulting tissue destruction would be caused by a local imbalance between proteinases and their inhibitors [7,37-42]. Recently the presence of urokinase-type plasminogen activator in squamous cell carcinoma and the absence in basal cell carcinoma was described, with the interesting finding that those squamous cell carcinomas that were lacking urokinase-type plasminogen activator were histologically well differentiated tumors [34,43].

Because not a single factor but cascades and combinations of different events is important in carcinogenesis, it is interesting to speculate on a possible role of SKALP/elafin in this process. SKALP/Elafin may interfere with elastolytic activity that tumor cells need to penetrate the dermal tissue either directly by inhibiting elastase [44,45] or indirectly through interference with an elastase-dependent pathway of plasminogen activation [46]. In addition, SKALP/elafin might inhibit unknown proteinases and interfere with proteolysis in an elastase-independent way. Therefore, within the spectrum of keratoacanthoma, actinic keratoses and squamous cell carcinomas with varying degrees of differentiation, a progressive loss of SKALP/elafin expression could facilitate invasive

growth. To date, a correlation between tumor aggressiveness and the expression of lytic enzymes such as heparanases, plasminogen activators, cathepsins, and metalloproteinases has been described (for review see references [7] and [47]). Furthermore, it has been shown that inhibitors of metalloproteinases or inhibitors of serineproteinases can block tumor cell invasion [1,48,49]. Most likely a panel of different enzymes and inhibitors will determine the migrational behavior of tumor cells, and SKALP/elafin might be part of this panel.

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Chapter 9

Demonstration of skin-derived antileukoproteinase (SKALP) and its target enzyme human leukocyte elastase in squamous cell carcinoma

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ABSTRACT

Skin-derived antileukoproteinase (SKALP), also known as elafin, is a strong and specific inhibitor of elastase and proteinase 3. SKALP is not present in normal epidermis, but is expressed by epidermal keratinocytes under hyperproliferative conditions such as psoriasis, wound healing, and in cell culture. In human epidermal tumours, SKALP is differentially expressed and restricted to tumours with distinct squamous differentiation. We have studied the presence of both SKALP and one of its known target enzymes, leukocyte elastase, in 41 squamous cell carcinomas of the skin. SKALP expression correlated with the degree of differentiation: strong expression was seen in well-differentiated cells and expression was absent in poorly differentiated tumour cells. Most of the squamous cell carcinomas showed elastase-positive cells in the tumour stroma and also within the tumour cell nests. SKALP may interfere with the proteolytic activity of infiltrating inflammatory cells or with hitherto unknown proteinases from the tumour cells. We hypothesize that in squamous cell carcinomas progressive loss of SKALP expression could facilitate tumour spread.

INTRODUCTION

Elastase, a major proteinase in humans, is predominantly present in neutrophils, although elastolytic activity has also been reported in monocytes, macrophages, smooth muscle cells, fibroblasts, and blood platelets [1]. Neutrophils contain 3 pg of elastase per cell, which results in a daily turnover of at least 250 mg elastase [2]. Human leukocyte elastase (EC 3.4.21.37) has a broad range of extracellular matrix substrates, such as elastin, proteoglycans, collagen, and fibronectin [1]. A significant role has been suggested for elastase in the pathophysiology of several diseases including lung emphysema, arthritis, nephritis, bullous dermatoses, sepsis, and the allergic late-phase asthmatic response [1,3]. Elastase can be very destructive if not controlled and the presence of enzyme inhibitors is one of the physiological mechanisms which protect

tissue against unwanted proteolysis [4]. Derangement of homeostasis between enzyme and inhibitor leading to tissue damage was first recognized by Laurell and Eriksson and assimilated in the concept of their protease-antiprotease imbalance hypothesis in emphysema [5]. This hypothesis is now generally accepted not only in emphysema, but also in cancer. Tissue destruction preceding invasion and metastasis is considered to be the result of a local imbalance in favour of the proteinase, and many proteinases and their inhibitors are associated with features of malignant behaviour [6,7].

Skin-derived antileukoproteinase (SKALP) is a low molecular weight, cationic, heat- and pH-stable serine proteinase inhibitor that specifically inhibits human leukocyte elastase and proteinase 3 [8-10]. Cloning and sequencing of the cDNA proved SKALP to be identical to elafin and elastase-specific inhibitor (ESI), as described by others [11-13]. SKALP is locally produced by epidermal keratinocytes under hyperproliferative conditions such as psoriasis, wound healing and keratinocyte culture [8,9,14]. Furthermore, SKALP is differentially expressed in human epidermal tumours [15].

The aims of this study were two-fold. First, we wished to determine whether SKALP expression is related to the degree of differentiation of tumour cells in squamous cell carcinoma (SCC). Second, because of the specific inhibiting properties of SKALP against human leukocyte elastase, we wished to study the presence of elastase-positive cells in SCC.

MATERIALS AND METHODS

Chemicals

Goat-anti-rabbit IgG biotin conjugate, avidin-alkaline phosphatase, and the chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, and aminoethyl carbazole were obtained from Sigma Chemicals, St. Louis, MO, U.S.A. Swine-anti-rabbit Ig conjugated with horseradish peroxidase (SWARPO) was obtained from Dakopatts, Glostrup, Denmark. Goat-anti-mouse Ig conjugated with alkaline

phosphatase (GAMAP) was obtained from TAGO, Burlingame, CA, U.S.A. Recombinant SKALP/elafin was a kind gift from Dr. Norman Russell, ICI Pharmaceuticals, U.K.

Tumours

Archival biopsy material used for routine histology was selected. The specimens had been fixed in buffered 4 per cent formalin for at least 24 h and had been embedded in paraffin. SCCs of various degrees of differentiation were selected, and 17 well-differentiated SCCs, 17 moderately differentiated, and seven poorly differentiated SCCs were used. Serial sections were employed for histopathological grading and for immunohistology.

Well-differentiated tumours were characterized by a high degree of differentiation, with polygonal squamous cells having abundant cytoplasm, well-developed intercellular bridges, and a large number of horn pearls, some with nearly complete central keratinization. Poorly differentiated SCCs showed tumour fields with completely atypical or anaplastic cells, with nuclear pleomorphism and hyperchromasia. Keratinization was almost completely absent and the tumour cells were devoid of clear intercellular bridges. Atypical forms of mitosis were seen. Tumours that showed some focal differentiation in addition of these features were classified as poorly differentiated. Those tumours that did not fulfil the criteria for the well- or poorly differentiated groups were classified as moderately differentiated SCCs.

Antibodies

A polyclonal rabbit antiserum against recombinant SKALP/elafin was raised as described before [9]. Control serum (preimmune serum) was drawn before the immunization procedure. The specificity of the antiserum was validated on Western blots and in functional assays as previously described [9].

A monoclonal mouse antibody to human neutrophil elastase was obtained from Dakopatts, Glostrup, Denmark.

Immunohistochemical double staining for SKALP and elastase

Before staining, sections were deparaffinized and preincubated with 10 per cent normal swine serum and 10 per cent normal goat serum in phosphate-buffered saline (PBS) for 15 min. This was followed by an incubation for 60 min with the polyclonal anti-SKALP serum and the monoclonal anti-elastase (at a dilution of 1:500 and 1:250, respectively) in PBS with 1 per cent bovine serum albumine (BSA). After washing in PBS, sections were incubated for 30 min with SWARPO and GAMAP (at a dilution of 1:50 and 1:20, respectively) in PBS with 1 per cent BSA and 5 per cent human AB serum. Subsequently, sections were washed in 100 mM NaCL, 5 mM $MgCl_2$ and 10 mM Tris in distilled water (AP buffer) and developed using a mixture of 0.33 per cent 5-bromo-4-chloro-3-indolyl-phosphate-toluidine and 0.66 per cent nitro-blue-tetrazolium in AP buffer. Thereafter, sections were washed in PBS and sodium acetate buffer and developed using aminoethyl carbazole as chromogenic substrate. Because of the purple precipitate due to the alkaline phosphatase in the double staining method, no haematoxylin counterstaining was performed. Finally, sections were washed in distilled water, dried, and embedded in glycerol-gelatin solution. In total, 36 of the 41 tumours were double-stained with antibodies against SKALP and elastase; the remaining five tumours were only stained with anti-SKALP antiserum. Haematoxylin and eosin (H&E) staining was carried out on all tumours.

Control staining was performed as indicated above, with the omission of monoclonal anti-elastase and the replacement of polyclonal rabbit anti-SKALP/elafin serum with preimmune serum of the same animal.

RESULTS

SKALP expression

Well-differentiated SCCs

The cells of all but one tumour showed SKALP expression, although the number of stained cells and the intensity of the staining varied. In

general, the staining intensity was most distinct in tumour fields with mature squamous epithelial cells that showed only slight atypia. Where horn pearls were present, SKALP expression was maximal in the region adjoining the keratinized center (Figs 1a and 1b).

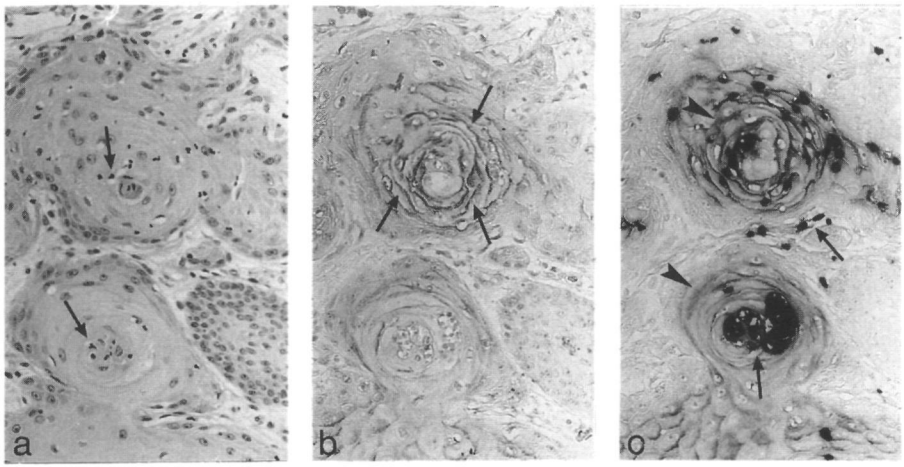

Figure 1. Serial sections of a well-differentiated SCC. (a). H&E staining. Tumour cell nests show a high degree of differentiation with nearly complete keratinization of the center. In the tumour cell nests PMN are visible (arrows). (b). Immunostaining with antibody against SKALP. Intensity of staining is maximal around the keratinized center. Note that SKALP staining is polarized and is present in the cytoplasm just beneath the cell membrane (arrows), in the direction of increasing differentiation, resulting in a rose-like appearance of the tumour cell nest. (c). Double staining with antibodies against SKALP and elastase. Elastase-positive cells are seen around and within the tumour cell nests (arrows), in close apposition to SKALP-positive cells (arrow heads).

Moderately differentiated SCCs

All but one tumour showed SKALP expression, which was maximal near areas of keratinization. Four tumours showed both positively and negatively stained tumour fields.

Poorly-differentiated SCCs

All the tumours containing fields with undifferentiated cells were negative for SKALP (Fig. 2). Some of the poorly-differentiated tumours

also showed cells with a higher degree of differentiation, resulting in SKALP-positive and SKALP-negative areas within the same tumour.

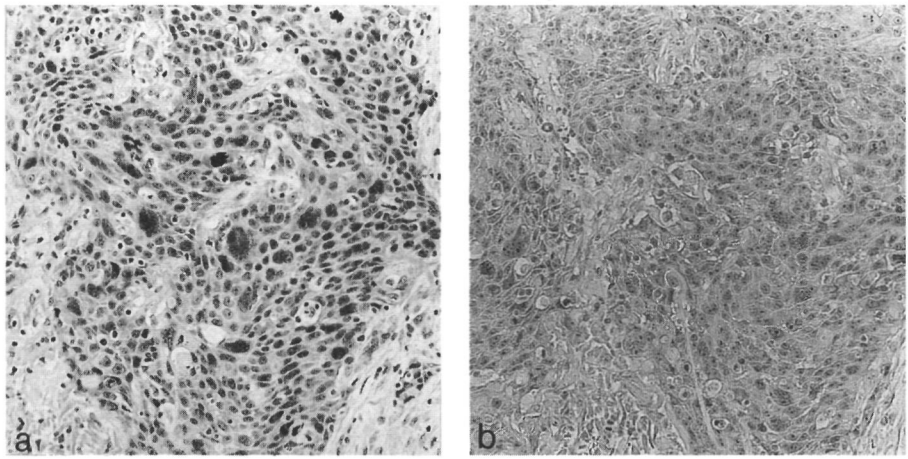

Figure 2. Serial sections of a poorly-differentiated SCC. (a). H&E staining. Atypical and anaplastic cells with basophilic cytoplasm are seen. Cells vary in shape, size and number of nuclei, some show abnormal mitotic figures. (b). Staining with anti-SKALP serum. No positive cells could be detected in this tumour area with poorly-differentiated cells.

Four of the seven tumours that were graded as poorly differentiated SCC showed SKALP expression in tumour areas with distinct squamous differentiation (Fig. 3).

In all grades of SCC, SKALP staining in positive tumour cells was cytoplasmatic, with a predominance towards the side of the cell in the direction of keratinization (Fig. 1), as has been previously reported in other types of tumours, in psoriasis and in wound healing.

Elastase-positive cells

In SCC, mixed infiltrates of mononuclear cells and polymorphonuclear cells (PMN) are often seen. In 32 of the 36 tumours that were double stained, elastase-positive cells could be demonstrated in all subgroups. The number of positive cells varied considerably between the tumours and within each tumour.

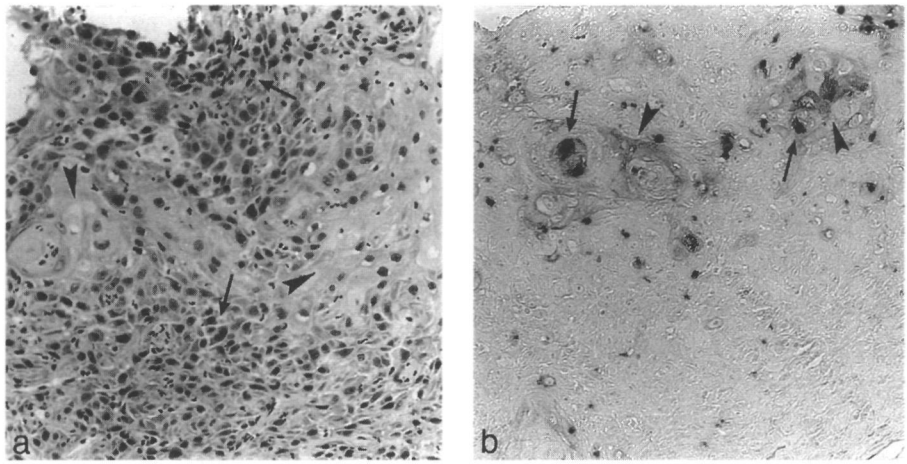

Figure 3. Serial sections of a poorly-differentiated SCC. (a). H&E staining. Tumour fields of basaloid cells are visible (arrows) together with tumour areas that show distinct squamous differentiation and keratinization (arrow heads). (b). Double staining with antibodies against SKALP and elastase. SKALP-positive cells are seen in the tumour fields that show keratinization. Elastase-positive cells (visible as the dark condensed spots) are seen around and within tumour fields (arrows), some in close apposition with SKALP-positive cells (visible as the more diffusely-stained cells, arrow heads). Note that basaloid cells are completely negative.

In 19 tumours, the elastase-positive cells were seen between and around SKALP-positive cells, being in close apposition (see Figs 1c and 3b). In Fig. 4, destruction of tumour fields by infiltrating cells is seen. The majority of infiltrating cells can be identified as PMN on H&E-stained sections (Fig. 4a). Double staining with anti-SKALP and anti-elastase antibodies shows that elastase-positive cells penetrate the tumour cell nests and appear to be in contact with the tumour cells (Fig. 4b). Groups of tumour cells appear isolated and individual cells are detached from the tumour mass. The infiltrating PMN are seen in both well-differentiated and poorly differentiated parts of the tumour (Fig. 5). Bearing in mind the functional relationship between SKALP and elastase, an interaction between SKALP and human leukocyte elastase in tumour cell nests is suggested.

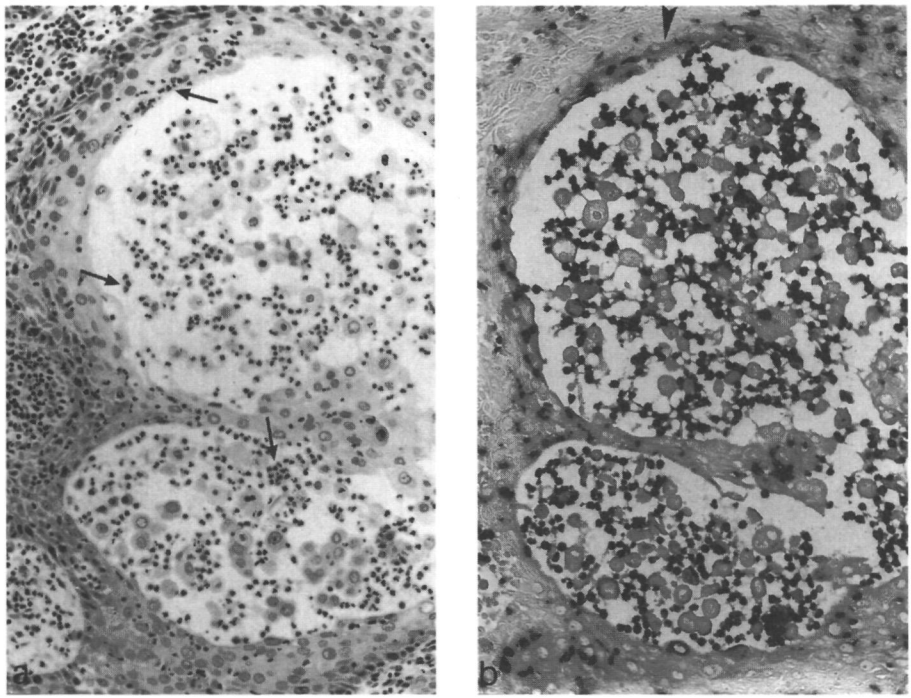

Figure 4. Serial sections of a poorly-differentiated SCC. (a). H&E staining. PMN are seen in close apposition to detached and lysed tumour cells in the center of the tumour nests. Note the great number of infiltrating PMN (arrows). (b). Double staining with antibodies against SKALP and elastase. Elastase-positive cells (visible as dark condensed spots) are seen adjacent and attached to isolated (groups of) tumour cells. SKALP presence is scarce, and restricted to one or two suprabasal, squamous cell layers at the edge of the tumour nest (arrow head).

In two of the 36 sections that were double stained, elastase-positive cells were present without any SKALP expression (6 per cent) and SKALP expression without the presence of elastase-positive cells was seen in three of the 36 doublestained sections (8 per cent).

DISCUSSION

In this study of SCC in human skin, we have demonstrated that the presence of the serine proteinase inhibitor SKALP correlates with the

degree of differentiation of the tumour cells. SKALP is exclusively expressed by well-differentiated cells in tumour areas that show distinct squamous differentiation.

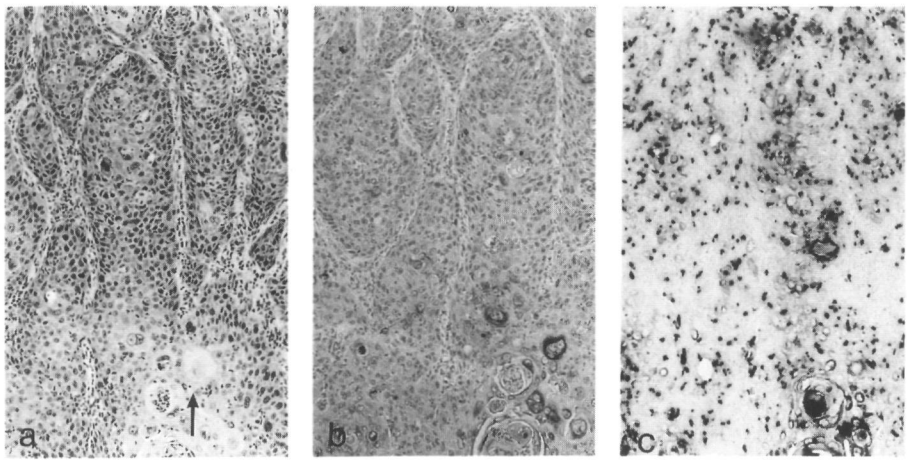

Figure 5. Serial sections of a poorly-differentiated SCC. (a). H&E staining. Tumour fields of basaloid cells are visible, and only scarce keratinization can be seen (arrow). (b). Immunostaining with antibody against SKALP. SKALP-positive cells are restricted to the well-differentiated part of the tumour (arrows). (c). Double staining with antibodies against SKALP and elastase. An enormous number of elastase-positive cells (visible as the dark condensed spots) can be seen in the tumour. Note that PMN are present in all parts of the tumour, irrespective of the differentiation grade of the cells.

In general, different degrees of malignancy may be present in different fields of the same tumour [16] and grading of the tumour is carried out according to the least differentiated area [17]. A relationship between histological differentiation and metastasis has been described, with poorly differentiated SCC of skin having a higher metastatic rate [18]. When SKALP expression was seen, irrespective of the degree of differentiation of the tumour, it was restricted to the well-differentiated cells. Cells positive for elastase, which is one of the target proteinases of SKALP, were found in 89 per cent of the tumours.

Inflammatory cells often accumulate at sites of tumour invasion, but reports about their relationship with tumour cells and tumour behaviour

are conflicting. Elastase-positive granulocytes were identified close to tumour cells and stroma of breast cancer tissue, where they might have a cytotoxic effect on the tumour cells and could play a protective role against tumour invasion and metastatic spread [19]. In contrast, an association between infiltrating cells and an increase of invasive growth of the tumour has also been described. In cutaneous tumours, infiltration of PMN that contain type IV collagenase, and extracellular deposition of the enzyme in the basement membrane zone resulted in more aggressive tumour behaviour [20]. Mast cells at sites of tumour invasion contributed to the connective tissue breakdown commonly associated with tumour invasiveness and metastatic spread [6]. PMN-induced detachment of cultured epidermal carcinoma cells from the substratum has been interpreted as a contribution of PMN to tumour cell killing, although the majority of the detached cells were alive [21]. In contrast, Sloane *et al.* suggested that the release of cathepsin B, which degrades pericellular protein, may contribute to detachment of the cells from the primary tumour and hence to metastasis [22].

The expression levels of proteinases have been implicated in tumour proliferation, tissue invasion and metastasis [6,7]. Because elastase is one of the target enzymes of the proteinase inhibitor SKALP, its expression in tumours was of particular interest to us. In cell culture, tumour cells of rat, mice, and humans have been proven to produce elastase or elastase activity [23-26]. Elastase might be used as an indicator of metastatic ability of Dunning R-3327 rat prostatic adenocarcinoma [27]; pharmacological interference reduces elastase activity and inhibits the metastatic spread of tumours in the lungs of mice [28]. Elastase may also facilitate tumour invasion in an indirect way, by activating plasminogen [29]. Finally, enhanced elastin degradation in patients with lung cancer [30], and breast cancer [31] has been reported, the elastin degradation being possibly due to proteolysis at the site of the tumour, mediated by elastase-like enzymes from tumour cells or infiltrating cells.

The balance between proteinases and proteinase inhibitors is considered to be of major importance in tumour invasion and metastasis, and many proteinase inhibitors are known to counteract the process of

tumour spread [6,7,32]. *In vivo*, a local increase of antiprotease and neutrophil elastase-α_1-proteinase inhibitor complexes in lung cancer has been reported [33]. SKALP is known to be produced by tumour cells, both *in vivo* and *in vitro* [15,34]. In normal skin, SKALP is not expressed; under hyperproliferative conditions, however, such as psoriasis, wound healing, and tumours, SKALP is found in suprabasal keratinocytes. In other words, SKALP is part of the regenerative/hyperproliferative differentiation programme, which also includes the expression of keratin 6 and 16 [35]. Teleologically, expression of SKALP could be interpreted as a mechanism to prevent the migration and invasive growth of (normal) keratinocytes under hyperproliferative conditions and during disturbance of the normal tissue integrity. According to this view, SCC cells that express SKALP would still be able to counteract invasive growth or detachment of tumour cells, by inactivation of elastase activity from infiltrating cells, stroma, or the tumour cells themselves. It remains to be investigated whether infiltrating elastase-positive cells promote or suppress tumour cell invasion and spread. In Fig. 4, a beneficial role of PMN is suggested because lysis of cells adjacent to PMN is seen. This putative cytotoxic effect of elastase-positive cells is in accordance with the findings of Schmitt *et al.* and Katayama *et al.* [19,21], but in contrast with effects of infiltrating PMN as described by Karelina *et al.* [20]. If elastase has a beneficial role with respect to tumour cell killing, then why is there a potent inhibitor simultaneously present? It is very unlikely that a proteinase inhibitor enhances tumour spread, since until now only anti-tumour effects have been described [6,7,32]. Speculatively, a physiological function of SKALP may be to control excessive elastase activity and to prevent damage to normal tissue. Moreover, SKALP in association with elastase, or fragments of SKALP after interaction with elastase, may be chemotactic for PMN, as has been described for α1-proteinase inhibitor [36]. Another possibility is that SKALP counteracts other proteinases present in tumour cells.

We would hypothesize that SKALP is one of the many factors that contribute to the maintenance of tissue homeostasis and the control of

tumour spread. Loss of SKALP expression during tumour progression from the well-differentiated SCCs to the more aggressive poorly-differentiated types, as observed in our study, could contribute to the altered migrational behaviour of the tumour. However, SKALP expression in itself may be insufficient, since cell-induced proteolysis has been described even in the presence of inhibitors [37,38]. Several mechanisms of inactivation of proteinase inhibitors have been reported, including oxidation by reactive oxygen metabolites and enzymatic degradation [39,40]. Cell-surface elastases may provide local proteolytic activity that is difficult to counteract [23,26]. For inhibition of its target enzyme, the proteinase inhibitor should be able to reach the active site of the proteolytic enzyme, since binding of the degrading enzyme to its substrate appears to be a very effective way of escaping inhibition [41]. Limited extracellular proteolysis at sites of contact may result in directional cell migration [42,43], and a balanced action of both proteinases and proteinase inhibitors might be involved in tumour cell invasion.

In conclusion, we have found a correlation between expression of a proteinase inhibitor and the degree of differentiation of tumour cells. Although this finding does not necessarily imply a causal relationship, it fits within a conceptual framework of tumour invasion and metastasis. At present, it is not clear whether SKALP only inhibits elastase released by infiltrating inflammatory cells or whether it also inhibits other still unidentified proteinases produced by tumour cells or stroma. The close apposition of SKALP and human leukocyte elastase in more than 50 per cent of the SCCs studied suggests a possible *in vivo* interference with the process of tumour spread. We do not know whether the lack of an absolute correlation in well-differentiated and moderately differentiated SCCs arises from the dynamics of PMN infiltration or from the ability of SKALP to counteract PMN migration. Future studies will be directed at elucidating the role of SKALP and its target proteinases in functional assays and in experimental models of tumour cell migration.

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Chapter 10

Summary, discussion and future prospects
Samenvatting
Dankwoord
Curriculum vitae

SUMMARY, DISCUSSION AND FUTURE PROSPECTS

The aim of this thesis as defined in the introduction was to characterize the SKALP molecule in greater detail, and to elucidate the (patho)physiological role of this proteinase inhibitor. Although after four years of research many questions with respect to the exact biological function of SKALP still need to be answered, the present thesis provides new data on its molecular aspects and relevance to the pathogenesis of various skin disorders.

In chapter 2 SKALP is characterized at the molecular level. It could be shown that SKALP is encoded for as a 117 amino acids protein, with a calculated molecular weight of 12.3 kDa, and consists of two functional domains, and a signal peptide of 22 amino acids. Cleavage of the signal peptide results in the 95 amino acids molecule, comprising the first domain with transglutaminase substrate motifs and a second domain with proteinase-inhibiting properties. Isolation of SKALP from cultured keratinocytes predominantly yielded this two-domain form, while in psoriatic scales the main form is the 6 kDa 57 amino acids form. The signal peptide points at the rough endoplasmic reticulum as the site for SKALP mRNA translation. In the near future a survey on subcellular SKALP localization will be performed. In the first domain, transglutaminase substrate motifs are found, which may point at another yet unknown function of SKALP. It was shown that SKALP can act as a transglutaminase substrate, although the exact cross-linking sites remain to be demonstrated. Both at the protein level [1,2], and at the mRNA level [3,4] SKALP and transglutaminase type 1 are present in the suprabasal compartment of the psoriatic epidermis. The co-expression of SKALP and transglutaminase in the tissue suggests but does not prove a functional interaction. The second domain with the proteinase-inhibiting property is not completely needed to inhibit human leukocyte elastase. The 9.9 kDa form (both the first domain with the transglutaminase substrate motifs and the second proteinase-inhibiting domain), the 6 kDa form (only the proteinase-inhibiting domain) and the 2.5 kDa form (fragment of the proteinase-inhibiting domain) all are functionally active

with respect to elastase inhibition.

In chapter 3 *in vivo* and *in vitro* studies on expression and induction are reported. Although data conflict [5-8], we are convinced that hardly any anti-elastase activity is present in normal skin. *In vivo*, a substantial induction of SKALP expression can be seen in hyperproliferative conditions such as inflammatory skin diseases, and wound healing. The time course of expression with a rapid onset of SKALP production by keratinocytes makes SKALP a locally produced 'acute phase reactant' [7,9]. *In vitro*, SKALP can be induced in different cell culture systems, provided that the media used contain serum factors. The cultured keratinocytes mainly produced SKALP in the 9.9 kDa molecule and other molecules of relatively high molecular mass, whereas low molecular forms of 6 kDa and smaller predominate in tape-stripped or psoriatic skin. On Western blots forms with a molecular mass between 9.9 and 6 kDa are also seen. Heterogeneity at the N-terminus probably resulting from proteolysis has been described for the 6 kDa form of SKALP [10]. In the different culture systems producing a multilayered epidermis, SKALP expression was restricted to the stratum granulosum and the outer layers of the stratum spinosum, in accordance with previous findings in psoriatic lesional or tape-stripped skin [11]. Different techniques yielded partly conflicting results regarding expression levels of SKALP in relation to grade of differentiation of the cultured keratinocytes, for which we do not have an explanation yet. The fact that SKALP is expressed in keratinocyte culture suggests that PMN presence is not required for induction of SKALP synthesis. Of course, SKALP production by keratinocytes and elastase release by PMN might be triggered by the same stimulus. Recently SKALP was reported to be induced in lung cell lines by the cytokines interleukin-1 beta (IL1-B) and tumor necrosis factor (TNF), and by the proteinases human neutrophil elastase and cathepsin G [9]. The experiments on regulation of SKALP expression in cultured keratinocytes will be extented in the near future.

In chapters 4 and 5, immunohistochemical localization studies are described. In psoriatic lesion, the expression of SKALP occurs exclusively in the upper layers of spinous and granular cells. Normal

skin, uninvolved psoriatic skin and basal cells in the psoriatic lesion did not show SKALP production. The presence of SKALP in serum and urine of psoriatic patients (chapters 6 and 7) shows that it must pass the basement membrane and reaches the dermal vessels, but on histology no SKALP presence can be observed in the dermis, probably due to the detection level. This might be due to the wash-out of extracellular SKALP during staining procedures. Another explanation for this discrepancy might be that SKALP in serum and urine is not skin-derived, but is the result of production elsewhere. As can be read in chapter 4, in some normal human tissues SKALP expression was observed. SKALP is expessed in normal tongue, gingiva, pharynx, tonsil, epiglottis, vocal fold, esophagus, vagina, uterine cervix, and the infundibulum of the hair follicle, which are all tissues that are in close relation with the outer world and are constantly exposed to inflammatory stimuli. When SKALP was expressed, inflammatory cells (PMN in particular) could often be observed histologically. Speculatively, SKALP might be expressed to limit proteolytic activity of PMN, thus guarding tissue integrity. In normal brain, cornea, larynx, lung, bronchus, duodenum, colon, liver, kidney, urethra, and skin of various locations no expression could be demonstrated. Histological findings were partly confirmed by Northern blot analysis. Findings in normal tissue suggest that the name SKALP is an unfortunate choice, since *skin-derived* only refers to the origin of SKALP in inflammatory skin disorders. SKALP is locally produced in response to mediators in early inflammation, and can be regarded as a true local 'acute phase reactant'. The local production in several tissues described in chapter 4 may explain SKALP concentrations in serum of normal controls (as compared to psoriatics) that are reported in chapter 7.

In the chapters 6 and 7, the application of SKALP as a tool for monitoring disease activity is described. In chapter 6 not only the presence of SKALP in urine of psoriatics has been demonstrated, but also a correlation with the severity of the disease. The most severe cases of psoriasis (erythroderma and pustular psoriasis) showed the highest urinary SKALP levels. Interestingly, besides free elastase, complexes of

SKALP with its target proteinase could be detected using a functional assay, which points at an *in vivo* interaction. However, initially we were not able to measure SKALP using a functional assay in the blood, which is the linking compartment between epidermis and urine, due to interference with other proteinase inhibitors in the circulation. Therefore an ELISA was established and used for measuring SKALP in serum and urine, as has been described in chapter 7. SKALP levels of serum rather than levels in urine correlated with the disease activity as represented by the PASI score. SKALP levels did not correlate with sex or age, and a circadian rhythm was not observed. It would be favorable if changes in SKALP level preceded clinical course, because then therapy could be started in advance. To study this, a daily measurement during a symptom-free period is indicated, and will be done in the near future.

For many years, the balance between proteinase and inhibitor has been thought to play a role in tissue destruction. The fact that leukocytes contain proteolytic enzymes which can be inhibited by normal serum was already described in 1905 by Opie [12]. In 1921 the same author reported that at the site of an inflammatory reaction anti-enzyme of the blood (antileukoprotease) serves to limit the activity of the leukocyte enzyme released, thus preventing extracellular proteolysis [13]. However, in 1964 Laurell and Eriksson were the first to link emphysema and α1-PI deficiency [14], and Dubertret discovered that in psoriasis a disbalance between proteolytic and antiproteolytic activity in favor of the former was responsible for tissue damage [15]. Proteinases causing tissue damage in several bullous diseases have been described by Takamori *et al.* [16]. Interestingly, several proteinase inhibitors have been described to be beneficial as they inhibit target enzymes in blistering diseases. In skin organ culture, plasminogen activator inhibitor 2 (PAI-2) was able to prevent acantholytic changes induced by pemphigus IgG [17]. In organ culture of normal human skin, the synthetic serine proteinase inhibitor camostat mesylate proved to inhibit blistering induced by dystrophic epidermolysis bullosa. Therapeutical use by topical application in patients with epidermolysis bullosa seemed promising [18]. Unfortunately, contact dermatitis was reported as a side-effect. Another

study reported successful treatment of atopic dermatitis with α1-PI [19]. Therefore, therapy based upon the use of proteinase inhibitors in (blistering) inflammatory skin diseases might be developed, and SKALP might be a candidate for therapeutical use.

In chapters 8 and 9, studies on tumor biology are reported. Since proteinases and their inhibitors play a role in tumor proliferation, invasion and metastasis [20-24], SKALP expression was studied in a number of epidermal tumors. Interestingly, SKALP appeared to be differentially expressed. The study described in chapter 8 proved SKALP to be present in squamous cell carcinoma, in Bowen's disease, in actinic keratosis, and in keratoacanthoma, but not in basal cell carcinoma. The absence of SKALP from basal cell carcinoma is in accordance with histological findings in psoriasis (chapter 5) and wound healing where SKALP is absent from basal cells [11]. The difference in expression in epidermal tumors suggests that SKALP can be used as a tumor marker. The expression of SKALP in squamous cell carcinoma appeared to be related to the grade of differentiation of the tumor. This was studied in a large number of squamous cell carcinomas, as is described in chapter 9. This survey showed a clear relation between SKALP expression and degree of differentiation of squamous cell carcinoma, SKALP being exclusively expressed in nests of well-differentiated tumor cells. Because of the heterogeneity of squamous cell carcinoma, some poorly differentiated squamous cell carcinoma did show SKALP expression, but in well-differentiated cells only. Elastase-positive cells were seen in all types of squamous cell carcinoma. A disadvantage of histological studies is the fact that they yield a static rather than a dynamic picture. The observation of SKALP in close proximity to elastase-positive cells is difficult to interpret with respect to *in vivo* interaction between SKALP and PMN. Is it beneficial for the patient because tumor cell detachment by PMN [25], possibly enhancing metastasis, is inhibited by SKALP? Or is it detrimental because PMN might be cytotoxic to tumor cells as has been described for macrophages [26]? Yet another option is that SKALP interferes with PMN migration, resulting in a prolonged presence of PMN in the tumor fields, thus enabling PMN to fulfill its anti-tumor

function better. Studies on effects of implantation of SKALP-expressing and SKALP-negative tumors in immunodeficient mice may be used to elucidate SKALP function in tumor biology. Also SKALP-deficient mice (knock-out for the SKALP gene) might contribute to a better understanding of SKALP action in skin cancer. In addition, effects of deletion or mutation of the SKALP gene may provide insight into the physiological function of SKALP. These studies are scheduled for the near future.

The observations described in this thesis have answered some of the questions raised at the start of the project. The exact biological function of SKALP is not known, although inhibition of PMN-derived proteinases suggests a role in the control of inflammation. However, the fact that SKALP can act as a substrate for transglutaminase is not to be ignored, and its biological relevance needs to be clarified yet. Clinically it may be used for monitoring severe psoriasis by measuring serum levels. Moreover it may, owing to its differential expression in human epidermal tumors, be used as a tumor marker. Local therapeutical use of SKALP as a treatment for downregulation of inflammation in some inflammatory skin diseases might be possible.

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SAMENVATTING

Ontsteking is de aspecifieke reactie van het lichaam om na een schadelijke prikkel weer tot herstel en evenwicht (homeostase) te komen. Bij dit ontstekingsproces zijn verschillende ontstekingsmediatoren betrokken, o.a. proteolytische (eiwitafbrekende) enzymen of proteïnasen, die zelf ook aanleiding kunnen geven tot weefselschade. Om zich te beschermen maakt het lichaam remmers tegen deze proteolytische enzymen, de zogenaamde anti-proteïnasen. SKALP (skin-derived antileukoproteinase) is een proteïnase remmer, die recentelijk door onze afdeling in de huid ontdekt werd.

Doel van het project was het nader karakteriseren van het SKALP molekuul, en het verruimen van het inzicht in de (patho)fysiologische rol van deze proteïnase remmer. Hoewel na 4 jaar nog niet alle van de in de introductie gestelde vragen beantwoord zijn, biedt dit proefschrift nieuwe gegevens wat betreft SKALP op molekulair niveau en wat betreft de betekenis van SKALP in de pathogenese van verschillende huidaandoeningen.

In hoofdstuk 2 wordt de karakterisering van SKALP op molekulair niveau beschreven. Het SKALP gen codeert voor een eiwit van 117 aminozuren, met een molekuul gewicht van 12,3 kDa en dat bestaat uit 2 functionele domeinen en een signaalpeptide van 22 aminozuren, dat duidt op translatie in het ruw endoplasmatisch reticulum. Na afsplitsing van het signaalpeptide resteert een molekuul van 95 aminozuren bestaande uit een eerste deel met transglutaminase substraat motieven en een tweede deel met proteïnase remmende eigenschappen. Isolatie uit gekweekte cellen levert voornamelijk het uit beide delen bestaande SKALP molekuul op, terwijl uit psoriatische huidschilfers vooral het tweede deel van 57 aminozuren verkregen wordt. De transglutaminase substraat motieven gelegen op het eerste deel kunnen duiden op een nog onbekende fuctie van SKALP. Aangetoond is, dat SKALP door transglutaminase als substraat 'gecrosslinked' kan worden, hoewel precieze plaats(en) waar transglutaminase aangrijpt nog niet bekend zijn. Dat in psoriasis transglutaminase en SKALP, zowel op eiwit niveau als

op het niveau van het messenger RNA, zich qua lokalisatie in hetzelfde (suprabasale) deel van de epidermis bevinden, duidt op een functionele interactie. Zowel het uit 2 delen bestaande SKALP molekuul met een gewicht van 9,9 kDa als het slechts uit het tweede deel bestaande deel met een moleculair gewicht van 6,0 kDa, als het kleinere fragment van dit laatste deel met een moleculair gewicht van 2,5 kDa zijn alle functioneel actief in het remmen van elastase.

In hoofdstuk 3 zijn *in vivo* en *in vitro* studies t.a.v. expressie en inductie van SKALP beschreven. Terwijl normaal vrijwel geen anti-elastase activiteit in de huid aanwezig is, wordt *in vivo* onder hyperproliferatieve condities, zoals huidziekten gepaard met ontsteking, en tijdens het herstel van beschadigde huid, SKALP geproduceerd. Omdat deze SKALP productie door keratinocyten snel op gang komt in reactie op een inflammatoire prikkel, kan SKALP met recht gezien worden als een 'acute fase eiwit'. *In vitro* kan, mits serum aan het kweekmedium is toegevoegd, SKALP door keratinocyten geproduceerd worden, en dan vooral de uit 2 delen bestaande grotere vorm van SKALP. In de meerlagige epidermis, zowel *in vivo* als *in vitro*, is SKALP vooral aanwezig in de meest gedifferentieerde (uitgerijpte) suprabasale en granulaire cellagen. SKALP expressie lijkt gerelateerd te zijn aan een verregaande differentiatiegraad, al zijn de verkregen resultaten niet geheel eenduidig. Waardoor de SKALP productie precies in gang gezet wordt, is nog niet duidelijk. Ontstekingsmediatoren maar ook proteolytische enzymen afkomstig van de polymorfkernige granulocyten (PMN) kunnen hiervoor verantwoordelijk zijn.

In de hoofdstukken 4 en 5 zijn de immunohistologische lokalisatie studies beschreven. Bij de psoriatische lesie blijkt SKALP in de bovenste, meest gedifferentieerde cellagen voor te komen. In normale huid en in basale cellen van de psoriatische plekken is geen SKALP zichtbaar. SKALP expressie blijkt niet beperkt tot de beschadigde of ontstoken huid, het komt ook voor in andere 'normale' weefsels. In de tong, het tandvlees, de achterwand van de keelholte, de amandelen, het strotteklepje, de ware stemband, de slokdarm, de vagina, de baarmoederhals, en een deel van het haarzakje komt SKALP voor.

Interessant is dat in deze weefsels, die alle in direct contact met de buitenwereld staan, een bepaalde mate van ontsteking als normaal beschouwd moet worden. Bij histologisch onderzoek werden ontstekingscellen gezien in de coupes, en SKALP zou aanwezig kunnen zijn om weefselbeschadiging t.g.v. deze ontstekingscellen te voorkomen. In hersenen, het hoornvlies, de overgang van de keelholte naar de luchtwegen, de longblaasjes, de luchtpijp, de dunne- en dikke darm, de lever en nier, de urinebuis en in de normale huid komt SKALP niet voor. Vooral dit laatste is opmerkelijk: '*skin-derived* antileukoproteinase', van de huid afkomstig... De naam is wat ongelukkig gekozen, en verwijst naar de plaats van productie in ontstoken- of beschadigde huid. De gegevens van het weefselonderzoek werden bevestigd door Northern blot analyse.

In de hoofdstukken 6 en 7 wordt gekeken of SKALP gebruikt kan worden als instrument om ziekteactiviteit te meten en te vervolgen. Hiervoor zijn concentraties van SKALP in urine en bloed gemeten. Deze concentraties bleken bij psoriasis patiënten veel hoger te zijn dan bij gezonde vrijwilligers of bij patiënten in een klachtenvrije periode. Verder bleken de concentraties in het bloed en de urine van de patiënten samen te hangen met de ernst van de huidziekte. Tijdens therapie, waarbij de psoriasis afnam, werd een daling van de SKALP concentratie in bloed en urine gezien. We concludeerden, dat het meten van SKALP concentraties inderdaad gebruikt kan worden om de ziekte-activiteit van psoriasis te vervolgen. Tevens is van belang dat SKALP-elastase complexen aangetoond werden in urine, wat aangeeft dat SKALP '*in vivo*' (dus niet alleen in een laboratorium of '*in vitro*' situatie) daadwerkelijk het proteolytische enzym elastase remt. Weefselafbraak t.g.v. een verstoring van het evenwicht tussen een enzym en zijn remmer is niet alleen bij ontstekingsprocessen, maar ook bij tumoren beschreven. Er wordt dan ook naar mogelijkheden gezocht om dit evenwicht tussen enzym en remmer, door het toedienen van een extra hoeveelheid remmer in de vorm van tabletten of zalf, te herstellen. SKALP zou een mogelijke kandidaat kunnen zijn om als therapie toegepast te worden.

In de hoofdstukken 8 en 9 zijn studies beschreven, waarbij de

aanwezigheid van SKALP in verschillende huidtumoren werd waargenomen. Proteolytische enzymen spelen een rol bij de groei van tumoren, bij de ingroei in omringend gezond weefsel en bij de doorgroei in bloedvaten, wat tot uitzaaiing van de tumor kan leiden. SKALP bleek aanwezig te zijn in sommige goedaardige tumoren en in die vormen van plaveiselcel carcinomen, waarvan de cellen goed gedifferentieerd waren. SKALP kan dan ook mogelijk gebruikt worden om tumoren te classificeren, wat belangrijk kan zijn bij het bepalen van de te volgen therapie. Direct contact tussen SKALP en PMN werd gezien in verschillende weefselcoupes van plaveiselcel carcinomen, maar de precieze betekenis hiervan is moeilijk aan te geven, vooral omdat op weefselcoupes een statisch en geen dynamisch beeld gezien wordt. Om de betekenis van SKALP in tumoren beter te begrijpen, zullen studies worden opgezet, waarbij tumoren met en zonder SKALP expressie worden ingebracht bij muizen zonder afweersysteem. Ook kan bij muizen het erfelijk materiaal wat codeert voor het SKALP eiwit uitgeschakeld worden om meer inzicht te verkrijgen in de fysiologische functie van SKALP.

Hoewel enige vragen gesteld in de introductie beantwoord zijn in dit proefschrift, is de precieze biologische functie van SKALP nog niet geheel bekend. De remming van enzymen afkomstig van PMN is suggestief voor een rol in de controle van ontsteking. Het feit dat SKALP ook als substraat voor transglutaminase kan dienen en dus betrokken kan zijn bij de vorming van de 'cornified envelope', de buitenste laag dode cellen van de huid, die het grootste deel van de huidbarrière functie vervult, kan duiden op een andere rol. Klinisch kan SKALP toegepast worden als maat voor ziekte-activiteit bij psoriasis en als tumor merkstof bij verschillende huidtumoren. Lokale therapeutische toepassing bij regulatie van ontsteking bij verschillende huidziekten behoort ook tot de mogelijkheden.

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CURRICULUM VITAE

Hans Alkemade werd op 18 juni 1959 geboren in Noordwijk. In 1978 deed hij zijn eindexamen gymnasium-B aan het Bonaventura-Kijckenborg te Leiden, waarna hij zijn studie geneeskunde startte aan de Katholieke Universiteit Nijmegen, die in 1988 werd afgerond.

Na als AGNIO anderhalf jaar gewerkt te hebben op poliklinieken allergologie in Dordrecht en Den Haag, begon hij zijn wetenschappelijk onderzoek op de afdeling Dermatologie van het Academisch Ziekenhuis Nijmegen St Radboud. Aanvankelijk als AGNIO, vervolgens vanaf 1 januari 1991 als AIO op het in deze thesis beschreven onderzoek betreffende de proteïnase remmer skin-derived antileukoproteinase (SKALP). In november 1993 werd hem een prijs toegekend in het kader van de Pharmacia Research Grant met als onderwerp 'Kwaliteit van zorg als resultaat van innoverend onderzoek', en liep hij de marathon van New York.

Per 1 januari 1995 zal zijn opleiding tot dermatoloog beginnen in het Academisch Ziekenhuis Nijmegen St Radboud.

STELLINGEN

behorende bij het proefschrift

SKIN-DERIVED ANTILEUKOPROTEINASE

Expression patterns and clinical applications

Hans Alkemade, Nijmegen, 16 december 1994

1. De serine proteinase remmer skin-derived antileukoproteinase (SKALP) is aanwezig in lesionale psoriatische huid en is een *in vivo* relevante remmer, aangezien zijn aanwezigheid (in gecomplexeerde vorm) in urine van ernstig aangedane psoriasis patiënten kan worden aangetoond. Dit proefschrift.

2. De SKALP concentraties in het serum van patiënten met ernstige psoriasis correleren met de klinische ernst van de psoriasis zoals weergegeven in de 'Psoriasis Area and Severity Index (PASI)', en kunnen gebruikt worden als graadmeter voor de ziekte-activiteit. Dit proefschrift.

3. SKALP kan gebruikt worden als een tumor merkstof vanwege zijn verschillende expressie in humane epidermale tumoren. Dit proefschrift.

4. Beauty is averageness. (Symons D. The evolution of human sexuality [Oxford University Press, 1979] / Langlois JH and Roggman LA. Attractive faces are only average. Psychol Sci 1990; 1:115-121).

5. Highly attractive facial configurations are not average, and preferences may exert a directional selection pressure on the evolution of human face shape. (Perrett DI, May KA and Yoshikawa S. Facial shape and judgements of female attractiveness. Nature 1994; 368:239-242).

6. De grootte van een probleem is afhankelijk van je standpunt, zodat het probleem kleiner lijkt naarmate je haar van verderaf bekijkt. De afstand Italië - Nederland is in dezen een redelijke.

7. De toegenomen waarde die aan mooie plaatjes bij wetenschappelijke artikelen gehecht wordt, leidt mogelijk tot een verschuiving van inhoud naar vorm. Dit is mijns inziens een slechte ontwikkeling, waarmee niet gezegd is dat ik ook maar iets tegen mooie vormen heb.

8. Door de waardering voor een afdeling en de toewijzing van (wetenschappelijk) personeel te koppelen aan de door die afdeling geproduceerde *output*, kan het publiceren verworden van middel tot doel.

9. De kwaliteit van een artikel wordt in het algemeen niet bepaald door eerstgenoemde auteur; ook hier geldt 'lest best' of, met enig gevoel voor understatement, 'last but not least'.

10. De oorzaak van teleurstelling moet niet in het resultaat gezocht worden maar in de verwachting.

11. Voetbal is een van de weinige denksporten waarbij een zekere mate van fysiek geweld is toegestaan.

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